Fatty Acid-Based Signalling in the Genus *Burkholderia*: Distribution, Mechanism, Cross-Linkage

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Everything becomes
a little different as soon as it is spoken out loud.
Hermann Hesse.

Summary

The genus *Burkholderia* comprises more than 90 species, which inhabit diverse ecological niches. They occur in water, soil, the plant rhizosphere and in association with fungi, insects and animals. Some *Burkholderia* show beneficial traits including biocontrol, bioremediation and plant growth-promoting properties. However, several *Burkholderia* species are also associated with plant, animal and human diseases. Members of the *Burkholderia pseudomallei* group are primary human and animal pathogens, causative agents of glanders and melioidosis. Members of the *Burkholderia cepacia* complex (Bcc) are pathogens in immunocompromised patients including those with cystic fibrosis and chronic granulomatous disease.

The expression of pathogenic traits is in many bacteria at least partially coordinated by specific cell-to-cell communication systems, a phenomenon commonly referred to as quorum sensing (QS). QS is a generic regulatory mechanism that allows bacteria to sense and respond to various self-generated signal molecules as a function of the population density. QS is very widespread among bacteria and it is a mechanism that allows intra- and interspecies, and even interkingdom communication. The clinical isolate *Burkholderia cenocepacia* H111, a member of the Bcc, employs two different QS systems. The first identified, and better characterized, QS system relies on *N*-**a**cyl-**h**omoserine **l**actones (AHLs). The second QS system utilizes the fatty acid derivative *cis*-2-dodecenoic acid (BDSF, ***B****urkholderia* **d**iffusible **s**ignal **f**actor). BDSF is synthesized by the enoyl-CoA-hydratase $RpfF_{Bc}$ and binds its cognate receptor RpfR. RpfR is a bifunctional enzyme that consist of PAS, GGDEF and EAL domains. Upon binding of BDSF, the phosphodiesterase activity of RpfR is stimulated, the intracellular c-di-GMP level is lowered and gene expression is altered. Both QS systems control a specific and overlapping set of genes in parallel.

This PhD project was designed to investigate i) the distribution of the BDSF-based QS system in the genus *Burkholderia* and other bacteria, ii) genomic factors relevant for BDSF signalling and iii) additional regulatory elements that affect the QS circuitry in *B. cenocepacia* H111. My investigations revealed that the BDSF-based QS system is present in various

Burkholderia species, including both pathogenic and environmentally beneficial strains. In addition, it was shown that *Cronobacter turicensis* also employs an active RpfF/R QS system to control biofilm formation and virulence. These findings show for the first time the widespread distribution of the RpfF/R system within both β- and γ-Proteobacteria. For these investigations I constructed a *cis*-2 fatty acid specific biosensor, which is sensitive to BDSF and related molecules in the nM range. Furthermore, my investigations shed light on the underlying mechanism of BDSF-regulated gene expression in *B. cenocepacia*. In the case of the stringently BDSF-controlled gene *bclA*, regulation was shown to occur via the 5' untranslated leader region. I could also provide evidence that BDSF- and AHL-dependent regulation is mediated via different promoter regions. Finally, a novel regulator, LepR (I35_4766), was identified, which was shown to be required for full expression of the lectins *bclACB* and for cepacian production. The LepR regulon partially overlaps the $RpfF_{Bc}$- and RpoN-regulon.

This thesis contributes to our understanding how expression of virulence determinants is regulated in the opportunistic human pathogen *B. cenocepacia*. This knowledge will lead to a better understanding of the pathogens' success in the environment and allows the identification of bacterial processes that might be targeted by anti-infective therapies.

Zusammenfassung

Der Genus *Burkholderia* umfasst mehr als 90 Spezies, die in verschiedensten ökologischen Nischen leben. Sie sind im Wasser, dem Erdboden, der Pflanzenrhizosphäre und in Assoziation mit Pilzen, Insekten und Tieren zu finden. Einige *Burkholderia* haben Merkmale, welche für den Menschen nützliche Eigenschaften sind; zur Bioremedation, der biologischen Bekämpfung von Pflanzenpathogenen und zur Förderung des Pflanzenwachstums. Allerdings werden auch mehrere *Burkholderia* Spezies mit Pflanzen-, Tier- und Humankrankheiten assoziiert. Mitglieder von der *Burkholderia pseudomallei* Gruppe sind primäre Human- und Tierpathogene, Verursachende von Malleus (Rotz) und Melioidose. Mitglieder vom *Burkholderia cepacia* Komplex (Bcc) können Krankheitserreger in immunsupprimierten Patienten, die z.B. an Zystischer Fibrose oder chronischer Granulomatose leiden, sein.

Die Expression von Pathogenitätsmerkmalen ist in vielen Bakterien zumindest teilweise durch ein spezifisches Zell zu Zell Kommunikationssystem reguliert, ein Ereignis welches als 'Quorum Sensing' (QS) bezeichnet wird. QS ist ein generischer Regulations-Mechanismus der den Bakterien ermöglicht, selbst-produzierte Signalmoleküle, als Funktion der Populationsgrösse, zu detektieren und entsprechend zu reagieren. QS ist unter den Bakterien weitverbreitet und QS ist ein Mechanismus der Kommunikation innerhalb derselben Art, zwischen Arten und zwischen den Reichen ermöglicht. Das klinische Isolat *Burkholderia cenocepacia* H111, Mitglied vom Bcc, hat zwei verschiedene QS Systeme. Das früher identifizierte und besser charakterisierte QS System basiert auf *N*-Acyl-Homoserin Lactonen (AHLs). Das zweite QS System basiert auf dem Fettsäure-Derivat *cis*-2-Dodecenoicsäure (BDSF) als Signalmolekül. BDSF wird durch die Enoyl-CoA-Hydratase $RpfF_{Bc}$ synthetisiert und bindet den Rezeptor RpfR. RpfR ist ein bifunktionelles Enzym, welches aus PAS, GGDEF und EAL Domänen aufgebaut ist. Wenn BDSF an RpfR gebunden ist, wird die Phosphodiesterase Aktivität von RpfR stimuliert, das intrazelluläre c-di-GMP Level wird reduziert und die Expression von Zielgenen wird reguliert. Beide QS Systeme regulieren parallel ein spezifisches und überlappendes Set von Genen.

Das vorliegende PhD Projekt wurde kreiert um Folgendes zu untersuchen; i) die Verbreitung vom BDSF-basierten QS System im Genus *Burkholderia* und anderen Bakterien, ii) genomische Faktoren die bedeutsam für die BDSF Signalwirkung sind und iii) zusätzliche regulatorische Elemente, die die QS Signalkaskaden in *B. cenocepacia* H111 beeinflussen. Meine Studie hat gezeigt, dass das BDSF-basierte QS System in verschiedensten *Burkholderia* Spezies vorkommt; in Pathogenen und umweltfreundlichen Stämmen. Zusätzlich wurde gezeigt, dass auch *Cronobacter turicensis* ein aktives RpfF/R QS System besitzt, welches Biofilm Bildung und Virulenz kontrolliert. Zum ersten Mal wird damit beschrieben, dass das RpfF/R System in β- und γ-Proteobakterien verbreitet ist. Die vorliegenden Resultate habe ich erreicht, mittels der Konstruktion von einem *cis*-2 Fettsäure spezifischen Biosensor, der nM Konzentrationen von BDSF und verwandten Molekülen detektieren kann. Im Weiteren konnte der BDSF induzierte Regulierungs-Mechanismus zumindest teilweise erklärt werden. Im Falle vom BDSF-regulierten Gen *bclA* funktioniert die Regulation durch die 5' nicht-translatierte Region. Ich konnte auch Hinweise bieten, dass die BDSF- und AHL-abhängige Regulation durch unterschiedliche Promoterregionen geschehen. Des Weiteren wurde ein neuer Regulator LepR (I35_4766) identifiziert, der für die Expression von den Lektinen *bclACB* und für die Cepacian Produktion gebraucht wird. Das Regulon von LepR überlappt teilweise mit dem $RpfF_{Bc}$- und RpoN-Regulon.

Die vorliegende Arbeit unterstützt das Verstehen, wie Virulenzfaktoren des opportunistischen Krankheitserregers *B. cenocepacia* H111 reguliert sind. Ein entsprechendes Wissen ermöglicht ein besseres Verstehen des Erfolgs vom Pathogen in der Umwelt und erlaubt die Identifizierung von bakteriellen Prozessen, welche bei Therapien zur Bekämpfung einer Infektion angegriffen werden können.

Contents

Chapter 1

Introduction

1.1 The genus *Burkholderia*

1.1.1 Taxonomy of the genus *Burkholderia*

Burkholderia cenocepacia are Gram-negative non-fermenting, motile rods of 1-2 μm in length, belonging to the genus *Burkholderia*. The genus *Burkholderia* is a group of ecologically and metabolically diverse bacteria, assigned to the β-subdivision of the phylum Proteobacteria. In 1950 the first isolates of *Burkholderia* were described as bacteria causing sour skin in onion bulbs, and at that time they were classified as *Pseudomonas* spp. (Burkholder, 1950). However, based on significant differences in 16S ribosomal RNA (rRNA) sequences of *Pseudomonas* spp., the new genus *Burkholderia* was defined in 1992 (Yabuuchi *et al.*, 1992). Members of the genus *Burkholderia* occur world-wide in various ecological niches including soil, water and in association with host organisms, including humans, animals, plants and fungi (Coenye and Vandamme, 2003; Compant *et al.*, 2008; Suárez-Moreno *et al.*, 2012).

Currently, the genus consists of almost 100 validly named species which are divided into two major lineages and an additional clade containing species of unique lines of descent (Depoorter *et al.*, 2016; Eberl and Vandamme, 2016). The first clade comprises human, animal and plant pathogens, including the *Burkholderia cepacia* complex (Bcc), the *Burkholderia pseudomallei* group, *Burkholderia glumae* and *Burkholderia gladioli*. Many species of this clade are pathogens, but conversely also have beneficial properties, as for example plant-growth promoting characteristics (Ho and Huang, 2015; Shehata *et al.*, 2016). The second clade consists of non-pathogenic, plant-beneficial-environmental (PBE) *Burkholderia* species, including the *Burkholderia xenovorans* group. PBE-strains have been identified in epiphytic, endophytic or endosymbiotic plant interactions, associated with fungi or insects, as well as free-living bacteria. Many species of this clade are associated beneficially with plants; many are able to fix nitrogen, to promote plant growth and to degrade recalcitrant compounds (Suárez-Moreno *et al.*, 2012). Thus, the potential of PBE-strains as biofertilizers and biocontrol agents or for rhizoremediation is often discussed (Eberl and Vandamme, 2016). The PBE clade was recently proposed to form a novel genus *Paraburkholderia* (Sawana *et al.*, 2014). Furthermore, there are the unique lineages *Burkholderia rhizoxinica, Burkholderia endofungorum* and *Burkholderia andropogonis*, which are not associated with the two main *Burkholderia* clusters. A phylogenic tree of 55 *Burkholderia* species, based on 16S rRNA gene sequences is given in Figure 1.1 (Eberl and Vandamme, 2016).

Figure 1.1 Phylogenetic tree of 55 *Burkholderia* species based on 16S rRNA gene sequences. The presence of the following phenotypic characteristics is indicated: antifungal activity, proteolytic activity and type III secretion. Dark boxes indicate presence; transparent boxes indicate absence of the characteristics. Red coloured species belong to the pathogenic group, green coloured species belong to the plant-beneficial and environmental group. Figure taken from Eberl and Vandamme (Eberl and Vandamme, 2016).

1.1.2 The *Burkholderia cepacia* complex

The *Burkholderia cepacia* complex (Bcc) is a group of 20 closely related species (De Smet *et al.*, 2015). Members of the Bcc are found in diverse environments, and have been isolated from a wide range of niches such as soil (predominantly the plant rhizosphere), from plants, water, animals and human clinical sources (Coenye and Vandamme, 2003). Many Bcc members have potential as biocontrol agents, as well as for the biodegradation of several pollutants, even though some Bcc members are plant pathogens (Burkholder, 1950; Parke and Gurian-Sherman, 2001). Generally, the biocontrol potential of Bcc members is attributed to the production of antifungal compounds (Schmidt *et al.*, 2009). The Bcc strains produce a wide range of secondary metabolites, including antibiotics (e.g. cepacin) and siderophores (Thomas, 2007). Some Bcc strains are able to fix nitrogen, to nodulate legumes and some have plant-growth promoting characteristics. However, the use of Bcc strains as biocontrol or plant-growth promoting agents has a significant limitation: In humans, Bcc bacteria have been associated with severe infections, especially in chronic granulomatous disease (CGD), cystic fibrosis (CF) and other immunocompromised patients (Speert, 2002; Mahenthiralingam *et al.*, 2008). CF is a serious inherited genetic disease and is caused by mutations in the gene encoding cystic fibrosis transmembrane regulator (CFTR), a transmembrane chloride ion channel (Matsui *et al.*, 1998; Boucher, 2007). The altered lung surface of CF patients, allows colonization by various opportunistic pathogens such as *Pseudomonas aeruginosa* and the Bcc bacteria, leading to severe chronic infections (Baldwin *et al.*, 2007; Cullen and McClean, 2015). *B. cenocepacia* and *Burkholderia multivorans* are the Bcc species most commonly isolated from CF patients (Mahenthiralingam *et al.*, 2008).

1.1.3 Virulence determinants of the *Burkholderia cepacia* complex

Bcc pathogens express various virulence factors that allow invasion of, and survival in the human host and cause disease (Loutet and Valvano, 2010). One of the most challenging characteristics of Bcc members is their intrinsic resistance to many commonly used antibiotics. The Bcc bacteria are resistant to antibiotic classes including aminoglycosides, polymyxins, quinolones, β-lactams and antimicrobial peptides (Leitao *et al.*, 2008; Sousa *et al.*, 2011). Therefore, it is extremely difficult to treat CF patients who are chronically infected with Bcc (George *et al.*, 2009). This multiresistance of Bcc members is due to the presence of various efflux pumps, the formation of biofilms that decrease the contact of antibiotics with the bacterial cell surface and due to changes in the cell envelope that reduce

the permeability of the membrane (Rushton *et al.*, 2013). All in all, to understand pathogenicity mediated by *Burkholderia*, a wide range of virulence determinants must be considered in the context of the strain and host (Loutet and Valvano, 2010)[1].

1.2 Quorum sensing: An intra- and interspecies communication system

Quorum sensing (QS) is an intercellular communication system that enables bacterial microorganisms to regulate gene expression at the cellular level in respect to the population density or growth state, respectively. By QS a population of microorganisms can cooperatively regulate biological processes contributing to antibiotic production, biofilm formation and other developmental programmes (Ryan and Dow, 2008). For instance, during host infection, QS allows bacteria to coordinate expression of virulence genes (Ham, 2013). This coordinated expression of virulence genes results in an increase of ecological fitness by saving cellular resources and avoiding early activation of the host defence system (Ham, 2013).

1.2.1 Mechanism and types of signal molecules

The basic mechanism of QS involves bacteria that synthesize, release and detect diffusible chemical signal molecules, designated as autoinducers: With increasing cell density, the concentration of autoinducers increases and when a certain threshold concentration is reached, bacteria respond to the diffusible signal molecules and alter gene expression (Fuqua *et al.*, 1994; Lewenza *et al.*, 1999; Whitehead *et al.*, 2001). Bacteria produce structurally diverse autoinducers, that are found in both Gram-negative and Gram-positive bacteria (Ryan and Dow, 2008). Gram-negative bacteria typically produce *N*-**a**cyl-**h**omoserine **l**actones (AHLs), fatty acid derivatives such as 3-hydroxypalmitic acid methyl ester and *cis*-unsaturated fatty acids (referred to as DSF, **d**iffusible **s**ignal **f**actor), cyclic dipeptides or Ax21, a small protein. Gram-positive bacteria, on the other hand, typically produce signalling molecules in the form of amino acids, modified peptides and occasionally fatty acid derivatives, for example γ-butyrolactones. Isomers of AI-2, a furanosyl borate diester were found in both Gram-negative and Gram-positive bacteria to be used as signalling molecules (Ham, 2013).

[1] Additional information on virulence determinants of the Bcc is available in the Appendix, Section A.

For each organism, the putative signal molecule must be distinguished from other bacterial metabolites. Winzer (Winzer, 2002) proposed four criteria to define cell to cell signal molecules: i) production occurs during specific stages of growth (required for QS signal molecules) or under certain physiological conditions or in response to changes in the environment; ii) extracellular accumulation and receptor specific recognition; iii) accumulation generates a concerted response upon reaching a critical threshold and iv) the cellular response extends beyond physiological changes required to metabolise or detoxify the signal itself.

Single bacterial species often possess multiple signalling systems, comprising related chemical structures (e.g. different type of AHLs in *P. aeruginosa* (Pearson *et al.*, 1995)), or unrelated signal molecules (e.g. AHLs and DSF in *B. cenocepacia* (Schmid *et al.*, 2012)). These signalling systems are in turn generally functionally interconnected with each other, either as hierarchical or parallel systems that regulate an overlapping set of genes (Malott *et al.*, 2005; Schmid *et al.*, 2012). Also often integrated in the signalling network are intracellular signalling systems (e.g. c-di-GMP based (Römling *et al.*, 2013)), encompassing a complex fine-tuned regulation of gene expression (Ryan *et al.*, 2006; Deng *et al.*, 2012).

1.2.2 Interspecies Communication

In nature, microorganisms are found in polymicrobial communities rather than in monoculture. Thus, an interaction between different species is required for cellular and community adaptation, development and maintenance (Ryan and Dow, 2008). Indeed, QS signal molecules enable interspecies and cross-kingdom communication.

Closely related bacterial species have been shown to produce identical or similar signalling molecules, suggesting a potential for interspecies communication (e.g. various *Xanthomonads* produce similar DSF type molecules (Deng *et al.*, 2011)). However, interspecies communication is also possible between species that do not produce the same signal molecules. The genomic presence of a functional signal receptor in the absence of production of the cognate signal is a widespread phenomenon (Ahmer, 2004; Ryan *et al.*, 2008).

Both Gram-negative and Gram-positive bacteria were found to modulate the morphological transition or biofilm formation of the eukaryotic yeast *Candida albicans*, presenting the possibility of inter-kingdom cross-talk (Wang *et al.*, 2004; Boon *et al.*, 2008; Davies and Marques, 2009; Vílchez *et al.*, 2010; Sztajer *et al.*, 2014).

1.3 Quorum sensing in members of the *Burkholderia cepacia* complex

The Section 1.3 has been published in 'Virulence' as 'Two quorum sensing systems control biofilm formation and virulence in members of the *Burkholderia cepacia* complex' by Suppiger *et al.* (Suppiger *et al.*, 2013).

1.3.1 AHL-based QS systems in the Bcc complex

All Bcc members encode at least one QS system that consists of homologs of the LuxR and LuxI proteins of *Vibrio fischeri*, where LuxI synthesizes an AHL signal and LuxR is an AHL receptor protein that activates or represses gene expression by binding to a consensus sequence (the so-called *lux* box) in the promoter regions of target genes (Waters and Bassler, 2005). AHL signal molecules can differ in the length and substitution of their acyl side chains. In many cases transcription of *luxI* is activated by the LuxR/AHL complex, providing a signal amplification mechanism via positive feed-back regulation (Shadel and Baldwin, 1992).

AHL production in the Bcc is strain-dependent with respect to both the quantity and the type of AHL molecules. Within the Bcc the CepIR QS system is fully conserved (Gotschlich *et al.*, 2001; Lutter *et al.*, 2001; Venturi *et al.*, 2004; Sokol *et al.*, 2007). CepI directs the synthesis of *N*-octanoyl-homoserine lactone (C8-HSL) and minor amounts of *N*-hexanoyl-homoserine lactone (C6-HSL; Figure 1.2) (Lewenza *et al.*, 1999; Gotschlich *et al.*, 2001). Epidemic strains of *B. cenocepacia* belonging to the ET12 lineage carry the *B. cenocepacia* genomic island (*cci*), which encodes an additional QS system named CciIR (Baldwin *et al.*, 2004). The AHL synthase CciI produces C6-HSL and minor amounts of C8-HSL, which are bound by the cognate receptor CciR. The CepIR and CciIR systems interact, with CepR positively regulating the expression of the *cciIR* operon and CciR negatively regulating *cepI* expression (Malott *et al.*, 2005). While CepR is mainly a positive regulator, CciR acts as a negative regulator of gene expression. In *B. vietnamiensis* strains, CepR is required for expression of yet another QS system (BviIR), which utilizes *N*-decanoyl-homoserine lactone (C10-HSL) (Malott and Sokol, 2007). Phenotypic assays as well as global transcript and protein analyses using *cepIR* and *cciIR* mutant strains have shown that AHL-mediated QS controls various functions, including swarming motility, biofilm formation and the production of virulence factors, such as proteases (e.g. the metalloproteases ZmpA and

ZmpB), siderophores, toxins and antifungal agents (Lewenza *et al.*, 1999; Huber *et al.*, 2001; Malott *et al.*, 2005; Tomlin *et al.*, 2005; Schmid *et al.*, 2012).

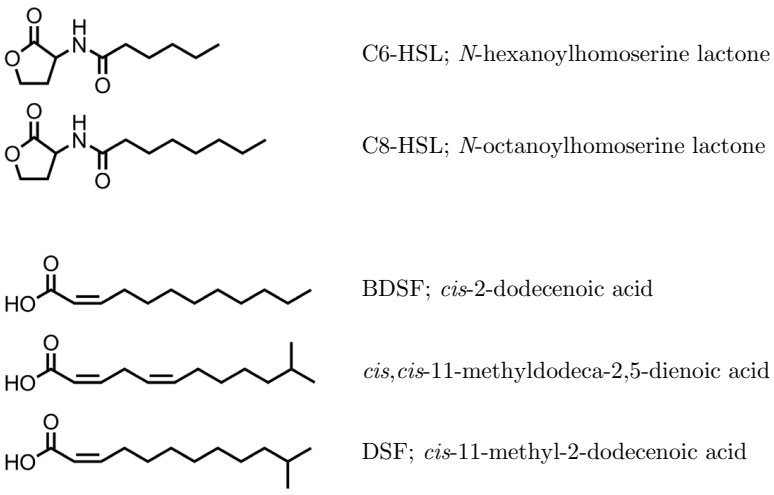

Figure 1.2 The different QS molecules produced by Bcc species. A, Signalling molecules in AHL-based QS. **B**, Signalling molecules in DSF family-based QS.

1.3.2 Fatty acid signal-based QS in the Bcc complex

In 2008, Boon *et al*. reported the identification of a novel fatty acid signal molecule that is produced by several *B. cenocepacia* strains (Boon *et al.*, 2008). The structure of the molecule synthesized by *B. cenocepacia* J2315 was identified as *cis*-2-dodecenoic acid, referred to as BDSF (***B**urkholderia* **d**iffusible **s**ignal **f**actor). BDSF is structurally related to DSF (**D**iffusible **s**ignal **f**actor, *cis*-11-methyl-2-dodecenoic), which was first isolated from supernatants of *Xanthomonas campestris* pv. *campestris* (Xcc) (Barber *et al.*, 1997; Wang *et al.*, 2004). In this plant pathogen DSF is activating the RpfCG two component system, leading to a lowered cellular c-di-GMP level and concomitantly to the expression of target genes involved in biofilm dispersal and virulence (He and Zhang, 2008; Deng *et al.*, 2011). DSF is synthesized by the gene products of *rpfF* and *rpfB* encoding a putative enoyl-CoA hydratase and a putative long-chain fatty acyl CoA ligase, respectively (Ryan and Dow, 2010; Deng *et al.*, 2011). In *B. cenocepacia* BDSF is synthesized by an RpfF homolog (BCAM0581 in *B. cenocepacia* J2315), named $RpfF_{Bc}$.(Boon *et al.*, 2008; Bi *et al.*, 2012) $RpfF_{Bc}$ is a bifunctional crotonase having both dehydratase and thioesterase activities, which enables the enzyme the direct conversion of the acyl carrier protein (ACP) thioester of 3-hydroxydodecanoic acid

into *cis*-2-dodecenoic acid (Bi *et al.*, 2012). BDSF accumulates in a cell density-dependent manner with maximum levels observed in the late stationary phase (Deng *et al.*, 2009). However, in contrast to the AHL-dependent CepIR system, the expression of the BDSF synthase is not subject to positive feedback regulation (Lewenza *et al.*, 1999; Deng *et al.*, 2009).

The BDSF-regulated QS system is involved in the control of several functions. Mutation of $rpfF_{Bc}$ resulted in decreased motility, reduced adherence to porcine mucin, diminished exopolysaccharides (EPS) production and lowered protease activity (Ryan *et al.*, 2009; Deng *et al.*, 2010; Deng *et al.*, 2012). In addition, the BDSF mutant strains were found to be more susceptible to antimicrobials and their ability to form biofilms was shown to be strongly reduced (McCarthy *et al.*, 2010; Udine *et al.*, 2013).

BDSF perception

Recent work has shown that the gene adjacent to $rpfF_{Bc}$ encodes the BDSF receptor protein RpfR (BCAM0580 in *B. cenocepacia* J2315) (Deng *et al.*, 2012). RpfR contains a GGDEF, an EAL and a PAS domain. PAS domains are able to bind a chemically diverse range of small-molecules, including hemes, flavins, di- and tricarboxylic acids, divalent metal cations, amino acids and coumaric acid (Henry and Crosson, 2011). RpfR and RV1364c, a σF regulatory protein of *Mycobacterium tuberculosis*, are the only proteins known to bind directly a fatty acid through their PAS domains (King-Scott *et al.*, 2011; Deng *et al.*, 2012). GGDEF and EAL are highly conserved domains involved in c-di-GMP turnover with diguanylate cyclase and phosphodiesterase activities, respectively (Römling *et al.*, 2013). Binding of BDSF to the PAS domain causes an allosteric conformational change of RpfR and thereby stimulates the c-di-GMP phosphodiesterase activity of the protein (Deng *et al.*, 2012). Consequently, in the presence of BDSF RpfR lowers the intracellular c-di-GMP level. Noteworthy, RpfR is the first example of a c-di-GMP metabolic enzyme that is directly activated by a QS signal molecule (Deng *et al.*, 2012).

The BDSF and AHL stimulons overlap

In order to identify BDSF-regulated genes, transposon mutagenesis, global transcript and protein profiling analyses were performed (Ryan *et al.*, 2009; McCarthy *et al.*, 2010; Schmid *et al.*, 2012). In *B. cenocepacia* J2315, $rpfF_{Bc}$ was found to regulate 372 genes with functions including motility, attachment, stress tolerance, virulence, transport, signal transduction,

multidrug resistance and detoxification (McCarthy *et al.*, 2010). Among these $RpfF_{Bc}$ dependent regulated genes, 65 were found to be also regulated by the putative BDSF sensor BCAM0227. The mapping of the *B. cenocepacia* H111 BDSF stimulon by RNA-Seq and shotgun proteomics confirmed BDSF-dependent regulation of genes known to be involved in biofilm formation and protease activity (Schmid *et al.*, 2012). Among the genes positively regulated by BDSF, many are known to be controlled by the CepIR QS system, suggesting that both signal molecules are required for full expression of some functions, including the large surface protein BapA and the EPS cepacian (Inhülsen *et al.*, 2012; Schmid *et al.*, 2012). However, the contribution of each of the systems in the regulation of target genes was found to be variable (Schmid *et al.*, 2012). While some genes are mainly regulated by either BDSF (e.g. *bclACB*) or AHLs (e.g. *aidA* or *cepI*), maximum transcription of *bapA* requires the presence of both signal molecules. On the basis of these results we proposed a model in which the BDSF and AHL QS systems operate in parallel to regulate specific as well as overlapping sets of genes (Schmid *et al.*, 2012) (Figure 1.3). BDSF-dependent signalling results in a reduction of the intracellular c-di-GMP levels, which in turn leads to differential expression of target genes (Deng *et al.*, 2012). Hence, it is very likely that an as-yet unidentified regulator (or regulatory cascade) responds to changes in the intracellular c-di-GMP level. Whether this regulator is independent of the CepIR system or the two QS regulatory cascades converge in one regulator remains to be elucidated.

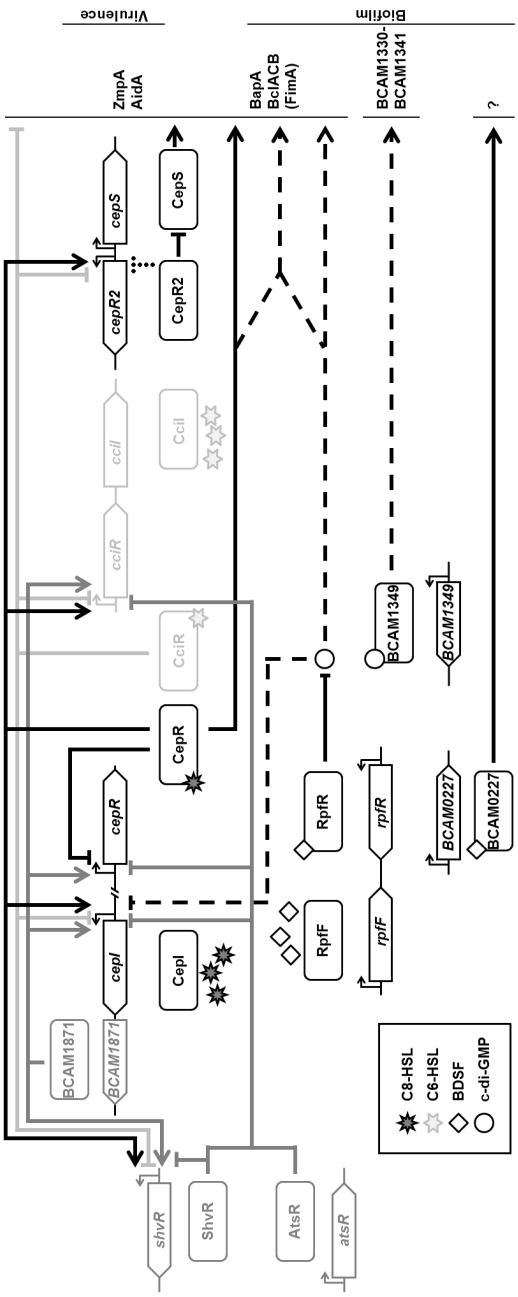

Figure 1.3 Model of the QS network in *B. cenocepacia*. The CepIR system consists of the AHL synthase CepI, which directs the synthesis of C8-HSL, and the transcriptional regulator CepR. The production of AHL signal molecules is fine-tuned by the activity of the regulators ShvR, AtsR and BCAM1871; however, the precise mechanisms how they affect AHL levels remain to be elucidated. The RpfFR system consists of $RpfF_{Bc}$, which directs the synthesis of BDSF, and its cognate receptor RpfR, which degrades c-di-GMP upon binding of BDSF. High intracellular c-di-GMP concentrations reduce the amount of AHL produced. The two QS systems operate in parallel to control a specific as well as an overlapping set of genes. The exact mode of action, by which altered c-di-GMP levels affect gene expression and if the two QS cascades converge, is currently unknown.

1.4 A quorum sensing-regulated factor: The lectin operon *bclACB*

1.4.1 Lectins

Lectins are glycan-binding proteins, or in other words, they are proteins that contain at least one non-catalytic domain that binds reversibly to a specific carbohydrate (Peumans and Van Damme, 1995). Even though there are diverse lectin structures, their sugar-binding activity is usually mediated by a single protein module, designated as the carbohydrate recognition domain (CRD). CRDs are built of characteristic structural folds, which in turn contain a sugar-binding site encoded by specific amino acid residues (Weis and Drickamer, 1996; Dodd and Drickamer, 2001).

Lectins can be differentiated based on their structure (Peumans and Van Damme, 1995); Monovalent lectins contain only one CRD, and are referred to as merolectins. Hololectins are multimeric proteins which contain two or more CRDs that are usually identical. By containing multiple binding sites, they are capable of mediating cell agglutination or precipitating glycoconjugates. The third lectin type are the chimerolectins, fusion proteins possessing a CRD and an unrelated domain possessing another biological activity (e.g. catalytic). Interaction of a lectin and its glycan ligand is generally based on hydrogen bonds and van der Waals forces between hydrophobic parts and aromatic amino acid side chains (Weis and Drickamer, 1996). Lectins often require divalent cations for their functionality, either to stabilize the binding site and to fix the positions of amino acids that interact with sugar ligands, or more rarely for direct coordination with the carbohydrate. Affinity of lectin-glycan interaction can be strongly increased by multivalent interactions, i.e. by clustering several binding sites and the formation of polypeptide oligomers (Weis and Drickamer, 1996).

Biological function of lectins

Lectins are proteins that are expressed in all domains of life, including yeast, vertebrates, invertebrates and plants (Dodd and Drickamer, 2001). Lectins that are intracellularly located have functions in trafficking, sorting and targeting of glycoproteins in the secretory and other pathways (Dodd and Drickamer, 2001). Lectins found outside the cell, either secreted or membrane bound, are generally involved in biological processes such as cell adhesion, endocytosis or modulation of cell signalling (Imberty and Varrot, 2008). The

ability of pathogens to induce infection is usually initiated by specific recognition of the host cell surface, its colonization and the delivery of virulence factors (Stones and Krachler, 2015). Unique structural characteristics for each cell type, tissue or species are provided for example, by glycoproteins or glycolipids that are located in cell membranes or glycocalyx, which can be targeted by lectins. Accordingly, extracellular lectins are involved in host-microbe interactions, by enabling attachment of viruses, bacteria or secreted proteins/ toxins to host cells, or by mediating a defensive role in plants and fungi towards viruses, bacteria or eukaryotes (Collins and Paulson, 2004; Kirkeby *et al.*, 2007). An example for the involvement of lectins in the delivery of virulence factors relies on the well characterized bacterial toxins of the AB class, including cholera- and shiga toxins (Sánchez and Holmgren, 2008; Beddoe *et al.*, 2010; Johannes and Römer, 2010).

1.4.2 Lectins in *Burkholderia cenocepacia* H111

In *B. cenocepacia* H111 three soluble lectins have been identified: BclA (I35_4184, 13.9 kDa), BclC (I35_4183, 28.3 kDa) and BclB (I35_4182, 25.6 kDa). The gene cluster I35_4184-82 is transcribed as an operon *bclACB* (Inhülsen *et al.*, 2012), which was found to be QS regulated by both AHL- and BDSF-based signalling systems (Inhülsen *et al.*, 2012; Schmid *et al.*, 2012). The lectins BclACB were shown to play a role in biofilm formation by contributing to its structural development (Figure 1.4) (Inhülsen *et al.*, 2012). This role may be related to the finding that the lectins BclACB are found on the bacterial cell surface and are likely to be secreted or released into the extracellular medium (Sulák *et al.*, 2011; Inhülsen *et al.*, 2012).

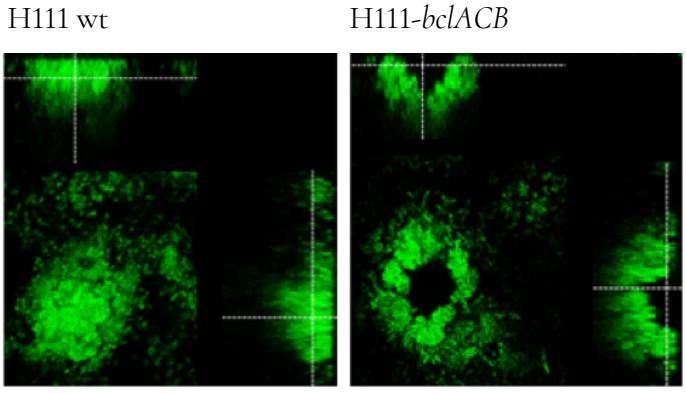

Figure 1.4 The lectins BclACB influence structural biofilm development in *B. cenocepacia* H111. Figure modified from Inhülsen and colleagues (Inhülsen *et al.*, 2012).

BclA, BclC and BclB share a similar mannose-specific C-terminal, that contains high similarity to LecB (PA-IIL) of *P. aeruginosa* (Lameignere *et al.*, 2008). LecB is a fucose-binding lectin and was shown to be involved in several virulence related functions, including pilus biogenesis, twitching motility, type IV, type II secretion and proteolytic activity, biofilm formation and epithelial cell attachment (Tielker *et al.*, 2005; Sonawane *et al.*, 2006; Chemani *et al.*, 2009).

BclA of *B. cenocepacia* H111 is a small protein built of 129 aa, that has 100 % similarity to BclA of *B. cenocepacia* J2315. BclA forms homodimers, with each monomer containing one sugar binding site which mediates Ca^{2+}-dependent specificity to D-mannosides (Lameignere *et al.*, 2008). Affinity of BclA towards its ligand mannose depends upon ligand density and interligand distance, demonstrated by binding of BclA to mannose functionalised glycol-nanoparticles (GNPs) (Reynolds *et al.*, 2013). Results indicate not only the presence of potentially multivalent ligands, but also the possibility of inhibiting bacterial adhesion by blocking the lectin-glycan interaction by using GNPs. BclA was also shown to bind heptose and heptose-containing oligo- and polysaccharides as present in the lipopolysaccharide (LPS) of *Burkholderia* species (Marchetti *et al.*, 2012). However, the purified protein BclA was only found to bind to truncated, rather than whole, LPS. Purified BclA of *B. cenocepacia* J2315 was found to bind and stimulate follicular lymphoma (FL) B-cells (Schneider *et al.*, 2015).

BclC of *B. cenocepacia* H111 shares 97.07 % amino acid identity with lectin BclC of *B. cenocepacia* J2315, which was recently purified and characterized in detail (Sulák *et al.*, 2010; Sulák *et al.*, 2011). BclC is a protein that contains two domains with distinct specificity. This is a characteristic that is rarely found among lectins, and therefore BclC is referred to as a superlectin (Sulák *et al.*, 2011). As mentioned previously, the BclC C-terminal domain has a Ca^{2+}-dependent specificity for mannose and heptose, similar to BclA. In contrast, the TNF-α-like N-terminal domain of BclC binds fucosylated oligosaccharides (Figure 1.5) (Sulák *et al.*, 2010). The protein BclC is found as hexameric structures, with dimeric C-terminal domains and trimeric N-terminal domains (Sulák *et al.*, 2011). The C-terminal domain was suggested to mediate mannose-dependent binding to the bacterial surface of *B. cenocepacia* (Sulák *et al.*, 2011). However, the surface molecule bound remains unknown. In the same study, it was shown that the BclC N-terminal domain induces carbohydrate-independent immunostimulatory activity in cultured respiratory epithelial cell cultures. In addition, the N-terminal domain of BclC was found to bind only undifferentiated induced pluripotent and embryonic stem cells and not differentiated stem cells (Tateno *et al.*, 2011).

Therefore, the purified N-terminal domain of BclC was used to target and distinguish specific cell types in various studies (Tateno *et al.*, 2014; Tateno *et al.*, 2015).

BclB of *B. cenocepacia* H111 also consists of a mannose-binding C-terminal domain with similarity to BclA (Lameignere *et al.*, 2008). The N-terminal domain of BclB does not contain similarity to any characterized protein (Sulák *et al.*, 2010). However, attempts were made at characterization of the protein (Wohlschlager, 2013). For example, BclB was found to exhibit strong toxicity towards the nematode *Caenorhabditis elegans*. Because the toxicity was found to be independent of mannose, it was suggested that this may be mediated via the N-terminal domain (Wohlschlager, 2013). However, the function and ligand of the BclB N-terminal domain remains unknown.

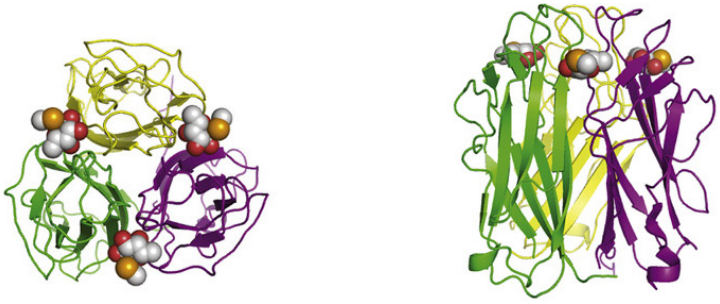

Figure 1.5 Crystal Structure of the trimeric lectin BclC N-terminal complexed with α-methylselenofucopyranoside (spheres). Figure taken from Sulák and colleagues (Sulák *et al.*, 2010).

Objective of the study

The aim of this PhD project was to analyse the BDSF-dependent signalling pathway, in particular its overlap with the AHL-dependent quorum sensing (QS) system, its regulatory mechanism and its occurrence in other bacterial species than *Burkholderia cenocepacia*.

This project was initiated, upon the BDSF receptor RpfR was identified and the overlap of the BDSF- and AHL-dependent QS pathway was described by our group and colleagues (Deng *et al.*, 2012; Schmid *et al.*, 2012). However, it was still unknown what regulatory components are required to converge the two QS cascades. In order to study the BDSF-dependent signalling pathway, the lectins BclACB were focused on. Expression of the lectins *bclACB* is tightly controlled by both BDSF- and AHL-based QS systems (Schmid *et al.*, 2012), the lectins BclACB were shown to be involved in biofilm formation (Inhülsen *et al.*, 2012) and they have a potentially relevant role in the physiology of *B. cenocepacia*.

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Chapter 2

Evidence for the widespread production of DSF family signal molecules by members of the genus *Burkholderia* by the aid of novel biosensors

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2.1 Summary

Many bacteria employ *cis*-2-unsaturated fatty acids, referred to as DSF family signals, to communicate with each other. Such systems have been shown to control biofilm formation, motility, production of hydrolytic enzymes and expression of virulence factors. We report the construction of novel biosensors on the basis of components of the BDSF-dependent circuitry of *B. cenocepacia* H111 and evaluated their utility for detecting the production of DSF family signal molecules. We show that a *luxAB*-based biosensor responds to nM levels of synthetic BDSF and is suitable to detect a wide range of *cis*-2 fatty acid molecules. Using this biosensor, we show that the production of DSF family molecules is widespread among members of the Bcc and demonstrate for the first time that DSF-based molecules are also produced by plant-associated *Burkholderia* species.

2.2 Introduction

Evidence has accumulated that many social behaviours of bacteria, including biofilm formation and the production of public goods, are regulated by quorum sensing (QS) (Fuqua *et al.*, 1994; Waters and Bassler, 2005; Galloway *et al.*, 2011). QS is a regulatory mechanism that allows bacteria to coordinate gene expression in a cell density-dependent manner. Such systems rely on the constitutive release of small diffusible signal molecules that accumulate in the environment. Only when a critical threshold concentration of the signal molecule is reached it interacts with a cognate receptor protein, resulting in the activation or repression of target genes (Whitehead *et al.*, 2001; Fuqua and Greenberg, 2002; Zhang and Dong, 2004). The most widespread signal molecules produced by Gram-negative bacteria belong to the family of *N*-acylhomoserine lactones (AHLs) (Whitehead *et al.*, 2001). However, many other structurally unrelated molecules have been identified that can serve as QS signals (Ryan and Dow, 2008; Deng *et al.*, 2011; Galloway *et al.*, 2011). Among these molecules are the *cis*-2-unsaturated fatty acids, often referred to as DSF (diffusible signal factor) family signals (Deng *et al.*, 2011). The first molecule of the DSF family, *cis*-11-methyl-2-dodecenoic acid, was identified in supernatants of the phytopathogen *Xanthomonas campestris* pv. *campestris* (Xcc) (Barber *et al.*, 1997). Subsequently, fatty acid-based QS-systems were also identified and characterized in members of the genera *Xylella*, *Burkholderia*, *Stenotrophomonas* and *Pseudomonas* (Fouhy *et al.*, 2007; Boon *et al.*, 2008; Davies and Marques, 2009; Beaulieu *et al.*, 2013). These DSF-dependent QS systems have been shown to control the production of

virulence factors and are thus essential for full pathogenicity of these organisms (Deng *et al.*, 2011).

Many strains belonging to the genus *Burkholderia* produce both AHL- and DSF-family molecules, which operate in parallel to regulate specific as well as overlapping sets of genes (Schmid *et al.*, 2012). Initial work showed that *Burkholderia cenocepacia* produces *cis*-2-dodecenoic acid, which was named BDSF (***B**urkholderia* **d**iffusible **s**ignal **f**actor) (Boon *et al.*, 2008). BDSF is synthesized by the enoyl-CoA hydratase $RpfF_{Bc}$ (Bi *et al.*, 2012) and is sensed by the receptor protein RpfR, which contains Per/Arnt/Sim (PAS)-GGDEF-EAL domains (Deng *et al.*, 2012). Upon binding of BDSF to the PAS domain, the c-di-GMP phosphodiesterase activity of RpfR is stimulated, which in turn lowers the intracellular c-di-GMP level. Global analyses of the *B. cenocepacia* H111 $RpfF_{Bc}$ regulon identified several BDSF-regulated genes, including the lectin operon *bclACB* and the large surface protein *bapA*. However, many of the identified genes were found to be maximally expressed only when both BDSF- and AHL-dependent QS systems are active (Deng *et al.*, 2012; Schmid *et al.*, 2012). Homologs of $RpfF_{Bc}$ and RpfR could be identified not only in many *Burkholderia* species, but also in strains belonging to the genera *Achromobacter*, *Yersinia*, *Serratia*, *Enterobacter* and *Cronobacter*, suggesting that fatty acid-based signalling as described for *B. cenocepacia* may be even more widespread than anticipated (Deng *et al.*, 2012). In order to detect DSF family molecules bioassays have been developed that are based on the restoration of endoglucanase production of a *rpfF* mutant of Xcc (strain 8523) or the induction of an *engXCA*::*gfp* or an *engXCA*::*gusA* gene fusion (Barber *et al.*, 1997; Slater *et al.*, 2000; Newman *et al.*, 2004; Wang *et al.*, 2004). The latter system was shown to be capable of detecting 0.5 μM DSF or 60 μM BDSF (Wang *et al.*, 2004).

Here, we describe the construction and characterization of novel *luxAB*- and *gfp*-based biosensors that respond to various *cis*-2-unsaturated fatty acids. The *luxAB* bioluminescent biosensor was found to be highly sensitive, detecting concentrations of BDSF as low as 10 nM. We used this novel tool to screen various *Burkholderia* strains for the production of *cis*-2 fatty acid signal molecules and show that its biosynthesis is particularly widespread among members of the *Burkholderia cepacia* complex (Bcc). We also show for the first time that DSF-based molecules are also produced by plant-associated *Burkholderia* species.

2.3 Results

2.3.1 Construction of a *luxAB*-based biosensor for the detection of DSF family signal molecules

In a recent study we have demonstrated that the *bclACB* lectin operon (I35_4184-82) is strongly BDSF-regulated, while the AHL-dependent CepIR system only marginally affects expression of this operon (Schmid *et al.*, 2012). Using the promoter region of the *bclACB* operon we engineered the sensor plasmid pAN-L15, on which the BDSF-dependent promoter drives the expression of the *luxAB* genes (Material and Methods S1). The sensor plasmid has a broad host-range and can easily be transferred to different bacteria via conjugation (Kovach *et al.*, 1995; Geiselhöringer, 1999). To test the suitability of the sensor plasmids, we introduced it into two different *B. cenocepacia* H111 genetic backgrounds: the *rpfF*$_{Bc}$ mutant H111-rpfF$_{Bc}$ and the *cepI rpfF*$_{Bc}$ double mutant H111ΔcepI rpfF$_{Bc}$. After addition of BDSF to the growth medium a significantly stronger induction response was observed when the sensor plasmid was in the H111-rpfF$_{Bc}$ background relative to the double mutant (Figure 2.1).

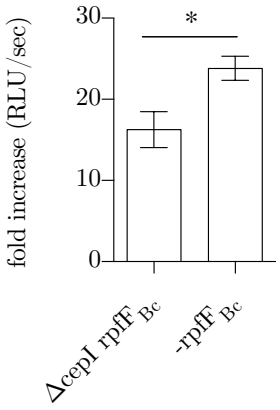

Figure 2.1 The genetic background influences sensitivity of the sensor plasmid pAN-L15. Plasmid pAN-L15 was introduced into the QS double mutant *B. cenocepacia* H111ΔcepI rpfF$_{Bc}$ or the *rpfF*$_{Bc}$ mutant *B. cenocepacia* H111-rpfF$_{Bc}$. Strains were grown in Luria-Berani (LB) medium (kanamycin 100 µg/ml) to an OD_{600} = 1.8 - 2.0, then diluted 1:1 in LB and supplemented with 10 µM BDSF (Sigma). Samples were incubated for 20 h at 30 °C and then assayed for bioluminescence as described in Material and Methods S 2.1. Fold increase is relative to the bioluminescence of the control without BDSF. Error bars indicate SEM, n=3. * P<0.05 (ANOVA, oneway, Bonferroni post-test with 95 % confidence interval).

Expectedly, no activation of the sensor was observed in an *rpfR* mutant background (Figure S 2.1). This is in agreement with our previous finding that for maximum expression of *bclACB* both the AHL- and the BDSF-dependent QS system have to be intact (Schmid *et al.*, 2012). We therefore used the H111-rpfF_{Bc} genetic background for subsequent experiments.

Previous work has identified BCAM0227 as an additional BDSF receptor in *B. cenocepacia* J2315 (McCarthy *et al.*, 2010). We therefore tested whether activity of the *bclACB* promoter on pAN-L15 is influenced by BCAM0227. However, in line with the previous finding that BCAM0227 is only involved in the regulation of a subset of the BDSF-regulated genes and does not control expression of the *bclACB* lectin operon in *B. cenocepacia* H111 (McCarthy *et al.*, 2010; Deng *et al.*, 2012) we did not observe any effect of this regulator on the functionality of the biosensor (Figure S 2.1).

2.3.2 The *luxAB*-based BDSF biosensor is sensitive to nM levels of BDSF

To test the sensitivity of the biosensor, various concentrations of synthetic BDSF were added to bacterial cultures of the reporter strain H111-rpfF_{Bc}/pAN-L15 and incubated for 20 h at 30 °C. Induction of luminescence was measured after addition of decanal, the luciferase substrate. Our results show that the biosensor responded to concentrations as low as 10 nM of synthetic BDSF, reaching a maximum level of bioluminescence with 100 nM of BDSF (Figure 2.2).

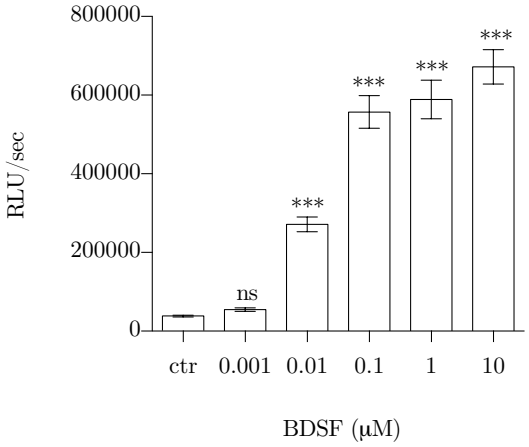

Figure 2.2 The *luxAB*-based biosensor H111-rpfF_{Bc}/pAN-L15 is highly sensitive for BDSF. The sensor strain was grown (as described in the figure legend of Figure 2.1) in the presence of BDSF with concentrations ranging from 1 nM to 10 μM; ctr, control (LB only). Error bars indicate SEM, n=3. ns, not significant; ** P<0.0001 (ANOVA, oneway, Dunnett's post-test with 95 % confidence interval).

2.3.3 The H111-rpfF$_{Bc}$/pAN-L15 biosensor responds to fatty acids with *cis*-2 configuration

To characterize the specificity of the H111-rpfF$_{Bc}$/pAN-L15 biosensor, we measured the induction of luminescence after the addition of molecules structurally related to BDSF as well as lauric acid and C8-HSL. These experiments showed that both DSF and *cis*-2-decenoic acid activated the biosensor at 1 µM, while no signal above the background was observed when other fatty acids or C8-HSL were tested at biological relevant concentrations of 10 µM and 200 nM, respectively (Figure 2.3A, Figure S 2.2). All active compounds share a *cis*-2 configuration. By contrast, *trans*-2-decenoic acid did not induce the biosensor (Figure 2.3). This is in line with the previous finding that the *cis*-2 configuration appears to be essential for the activity as a signal and for binding to the receptor protein RpfR (Wang *et al.*, 2004; Deng *et al.*, 2012).

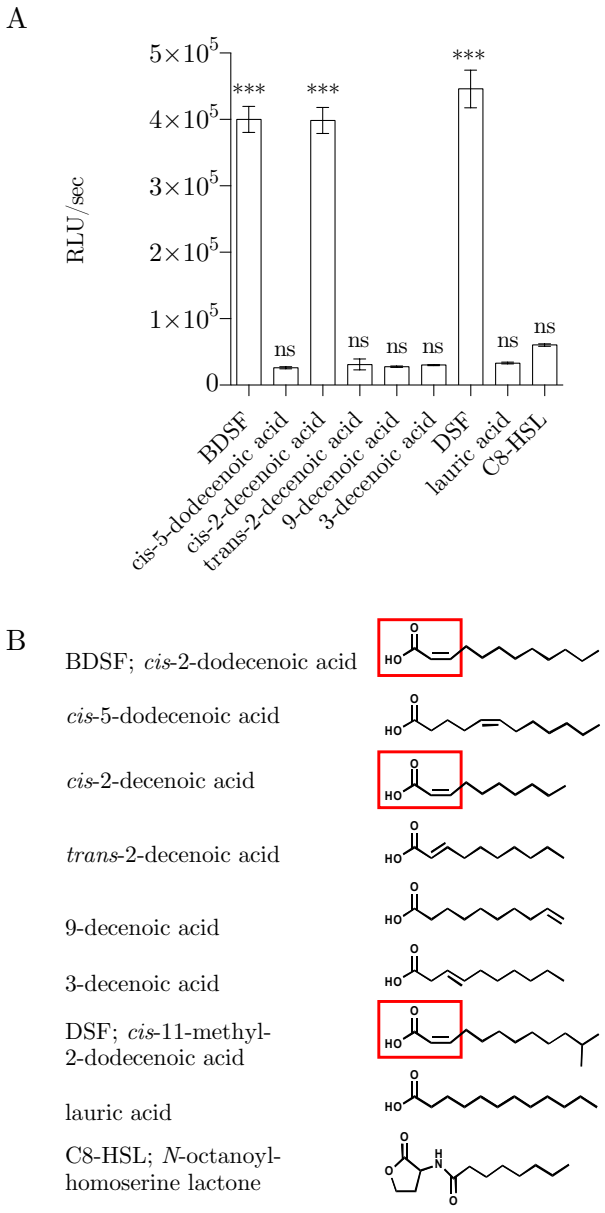

Figure 2.3 The sensor plasmid pAN-L15 is responsive to various fatty acids with a *cis*-2 configuration. A, The sensor strain *B. cenocepacia* H111-rpfF$_{Bc}$/pAN-L15 was grown in LB medium supplemented with different *cis*-2 fatty acids (10 µM, Sigma), lauric acid (10 µM, Sigma) or C8-HSL (200 nM, Sigma). Error bars indicate SEM, n=3; ns, not significant; *** P<0.0001 (ANOVA, oneway, Bonferroni post-test with 95 % confidence interval). **B**, Chemical structures of the tested compounds; the *cis*-2 configuration of respective fatty acids is depicted in a red box.

2.3.4 Production of DSF family signals is widespread among members of the Bcc

We next evaluated the suitability of the biosensor to detect the production of *cis*-2 fatty acids by bacteria in simple cross-streak assays. We cross-streaked the wild type *B. cenocepacia* H111, the *cepI* mutant H111ΔcepI, the *rpfF*$_{Bc}$ mutant H111-rpfF$_{Bc}$ and the double mutant H111ΔcepI rpfF$_{Bc}$ against the biosensor. Signals could only be detected for the wild type and the *cepI* mutant, suggesting that the biosensor does not cross-react with the AHL-dependent QS system (Figure 2.4A). We also investigated whether the biosensor could be used for the detection of *cis*-2 fatty acid signal molecules in culture supernatants. To this end, cell free culture supernatants were added to cultures of the biosensor and bioluminescence was measured in a plate reader (Synergy HT; Bio-Tek, Germany) after 20 h incubation. In agreement with the results obtained from the cross-streak experiments, the biosensor was significantly activated in the presence of supernatants of strains synthesizing BDSF but not by the supernatant of H111-rpfF$_{Bc}$ or H111ΔcepI rpfF$_{Bc}$ (Figure 2.4B).

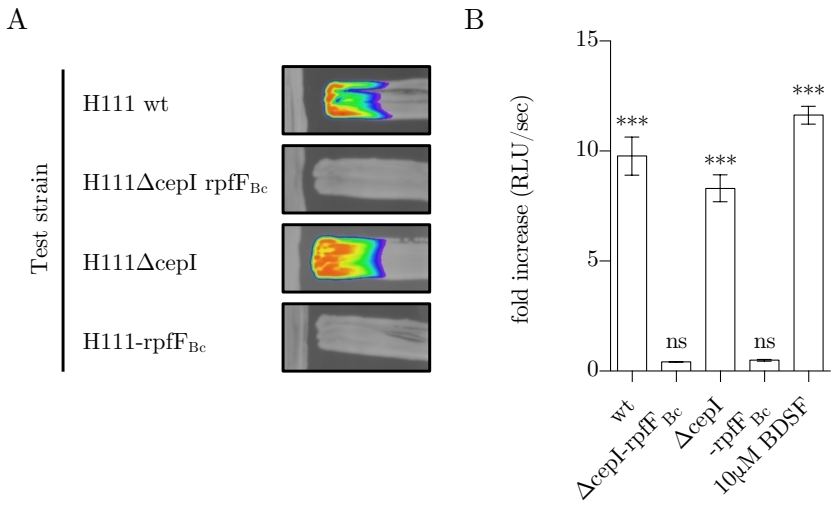

Figure 2.4 Activation of the *B. cenocepacia* H111-rpfF$_{Bc}$/pAN-L15 biosensor. A, The biosensor strain (horizontal) and test strains (vertical) were cross-streaked on LB agar plates as described in Material and Methods S 2.1. Only strains producing BDSF (*B. cenocepacia* H111 and H111ΔcepI) induced reporter gene expression. **B**, The sensor strain *B. cenocepacia* H111-rpfF$_{Bc}$/pAN-L15 was grown for 20 h at 30 °C in LB medium supplemented 1:1 with supernatants of the *B. cenocepacia* H111 wild type, the *cepI rpfF*$_{Bc}$ double mutant, the *cepI* mutant H111 ΔcepI and the *rpfF*$_{Bc}$ mutant H111-rpfF$_{Bc}$ or BDSF (10 µM). Samples were assayed for bioluminescence as described in Material and Methods S 2.1. Fold increase is relative to reporter gene induction in medium only. Error bars indicate SEM, n=4. ns, not significant; *** P< 0.0001 (ANOVA, oneway, Dunnett's post-test with 95 % confidence interval).

A Blast search identified $RpfF_{Bc}$ and RpfR homologs in all available *Burkholderia* genome sequences (Table S 2.1) (Winsor *et al.*, 2008), suggesting that the production of DSF-family signals may be widespread within this genus. Cross-streak and liquid culture assays were performed to test various strains for the production of DSF family signal molecules. Under the conditions tested, we found that production of DSF family signals is common among all members of the Bcc (the novel species *Burkholderia pseudomultivorans* was not tested) (Table S 2.1). *Burkholderia. sordidicola* (LMG22029), a strain outside of the Bcc, showed production of a DSF-family signalling molecule in both liquid and cross-streak experiments. *Burkholderia caledonica* (LMG19076), *Burkholderia graminis* (LMG18924), *Burkholderia phenoliruptrix* (LMG22037), *Burkholderia terricola* (LMG20594) and *Burkholderia plantarii* (LMG9035) induced the biosensor in cross-streak experiments but not significantly in liquid culture, suggesting condition-dependent production of DSF-based signalling molecules (Figure S 2.3, Table S 2.1).

2.3.5 Construction of a *gfp*-based BDSF biosensor for single cell, real time investigations

Previous work has shown that the BDSF-dependent QS system of *B. cenocepacia* controls biofilm formation (Deng *et al.*, 2012; Schmid *et al.*, 2012). To visualize the production of BDSF within biofilms, we constructed a transcriptional fusion of the *bclACB* promoter with an unstable variant of the green fluorescent protein (GFPmut3*), yielding plasmid pAN-G3. The functionality of this sensor plasmid was confirmed by cross-streak experiments against different *B. cenocepacia* strains (Figure S 2.4). Next, we transferred the sensor plasmid into the wild-type *B. cenocepacia* H111 and used this transgenic strain to monitor the spatial and temporal production of BDSF during biofilm development in a flow-through chamber system. In this setup, fluorescent cells were first observed when microcolonies were formed (approximately 24 h after inoculation) and at this time point only very few fluorescent cells were found outside of microcolonies (Figure 2.5). In 48 h old biofilms, virtually all cells within the microcolonies were fluorescent and many induced cells were also observed outside the microcolonies. These results indicate that a critical population density is required to trigger the QS response, an expression pattern that is compatible with an expected BDSF-dependent cross-induction of cells within microcolonies. In order to exclude the possibility that activation of the biosensor occurs by routes other than the BDSF-dependent QS system in biofilms, we also transferred the biosensor into the $rpfF_{Bc}$ mutant as well as into the *cepI* $rpfF_{Bc}$ double mutant and monitored fluorescence during biofilm

development. In agreement with previous work (Deng *et al.*, 2012; Schmid *et al.*, 2012) we observed that these strains formed less and structurally different biofilms. More importantly, fluorescence was greatly reduced in the biofilm of the *rpfF*$_{Bc}$ mutant and was found to be further reduced in the biofilm of the *cepI rpfF*$_{Bc}$ double mutant (Figure S 2.5). Exposure of a 48 h old biofilm of H111ΔcepI rpfF$_{Bc}$ to 10 µM BDSF for 3 h markedly increased fluorescence. In conclusion, these experiments strongly support a model in which the RpfFR system is the major regulator of *bclACB* expression while the CepIR system only plays a subordinate role.

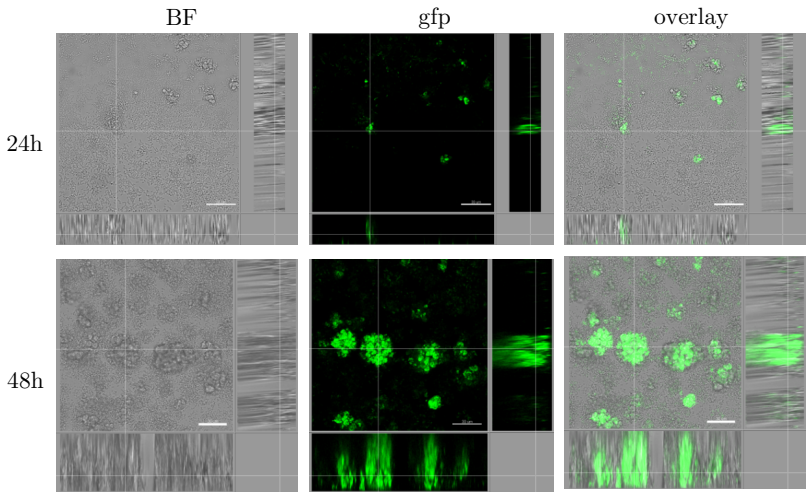

Figure 2.5 Visualization of BDSF production in a *B. cenocepacia* biofilm. Biofilms of *B. cenocepacia* H111 harbouring the *gfp*-based sensor plasmid pAN-G3 were cultivated in a flow chamber system as described previously (Inhülsen *et al.*, 2012). After 24 and 48 h the biofilms were inspected with a confocal laser scanning microscope (Leica, DM 5500 Q, Wetzlar, Germany) with a 40 x 1.15 oil objective. Data were analysed with Leica Application Suite (Mannheim, Germany) and the Imaris 7.7.2 software package (Bitplane, Zurich, Switzerland). Green fluorescence is indicative of BDSF production. BF, bright field; scale bar: 30 µm).

2.4 Conclusions

Evidence has accumulated over the past years that many Gram-negative bacteria are able to synthesize and respond to fatty acid signal molecules (Boon *et al.*, 2008; Deng *et al.*, 2011). This class of signals has been shown to play important roles in intraspecies, interspecies and even interkingdom communication (Boon *et al.*, 2008; Deng *et al.*, 2010; Alavi *et al.*, 2013; Deng *et al.*, 2013; Ryan *et al.*, 2015). At present it is not entirely clear how widespread the production of these signals is, but a bioinformatic analysis revealed that RpfF homologs are present in many bacterial genomes (Deng *et al.*, 2012). The biosensors developed in this study will facilitate the identification of the respective signal molecules and provide a tool for investigating the underlying signalling pathways. We show that the *luxAB*-based biosensor is very sensitive to BDSF (as low as 10 nM), but is also activated by other fatty acids containing a *cis*-2 configuration, which was previously shown to be critical for biological activity (Wang *et al.*, 2004). Although the GFP version of the biosensor is less sensitive, we show that it is a highly valuable tool for single cell, real time studies. It is worth noting that both sensor plasmids have an origin of transfer as well as a broad-host origin of replication and therefore can be transferred to a wide range of bacteria (Kovach *et al.*, 1995; Geiselhöringer, 1999).

In this study we have employed the biosensor to survey various *Burkholderia* species for the production of DSF family signal molecules. In agreement with the available bioinformatic information we found that all tested strains of the Bcc were capable of activating the biosensor. The Bcc is a group of 18 closely related species, which are notable for their ability to metabolize a wide range of organic compounds and to thrive in many different environments (Peeters *et al.*, 2013). However, they are also known as opportunistic pathogens that are capable of infecting immunocompromised patients or individuals suffering from cystic fibrosis (CF). This analysis extends previous work that showed, by using high-performance liquid chromatography (HPLC) and mass spectrometry, that BDSF is not only produced by *B. cenocepacia* but also by 8 additional species of the Bcc, namely *B. ambifaria*, *B. anthina*, *B. dolosa*, *B. lata*, *B. multivorans*, *B. pyrrocinia*, *B. stabilis*, and *B. vietnamiensis,* with some of these species synthesizing additional molecules, such as *cis*,*cis*-11-methyldodeca-2,5-dienoic acid or DSF (Deng *et al.*, 2010). Here we show for the first time the production of DSF family signals by *Burkholderia* strains that belong to a cluster of approximately 30 species that appear to be exclusively associated with plants and are beneficial for their hosts (Compant *et al.*, 2008; Gyaneshwar *et al.*, 2011; Suárez-Moreno *et*

al., 2012). Previous work has demonstrated that the RpfFR system in *B. cenocepacia* is essential for virulence in different infection hosts (Deng *et al.*, 2012). Given that members of the beneficial *Burkholderia* clade are not pathogenic it will be of great interest to unravel the functions regulated by DSF signals in these bacteria.

Acknowledgments

We thank I. Scholl for technical assistance. This work was supported by the Swiss National Science Foundation (project 31003A_ 143773).

2.5 Supporting Information

2.5.1 Material and Methods S 2.1

Bacterial Strains, Plasmids and Growth Conditions

Strains and plasmids used in this study are listed in Table S 2.2. Unless otherwise stated, strains were grown aerobically at 37 ºC in LB Lennox broth (Difco). Antibiotics were used at the concentrations (in μg/ml) indicated in parentheses: for *E. coli*, ampicillin (100), kanamycin (25), gentamicin (10) and chloramphenicol (30); and for *B. cenocepacia*, kanamycin (100), gentamicin (20) and chloramphenicol (80).

Plasmid Construction

A 653 bp promoter region of the *bclACB* operon was PCR amplified using *B. cenocepacia* H111 genomic DNA (gDNA) as template and the appropriate primers (PbclA_BamHI_F: 5'-GGATCCAGTCGTAGCGAGAAGAGGA-3' and PbclA_BamHI_R: 5'-GGATCCTTGAGAATCAGCCATGCTT-3'). The DNA amplicons were cloned into pGEM®-T Easy vector (Promega), according to the manufacturers protocol and transformed into *Escherichia coli* DH5α. After purification of the plasmid DNA using QIAprep® Spin Miniprep Kit (Qiagen) the plasmids were digested with the restriction endonuclease BamHI-HF. The DNA fragment was ligated into the corresponding site of the vector pGA-L14 or pGA-G2 (Kovach *et al.*, 1995; Geiselhöringer, 1999), generating pAN-L15 or pAN-G3, respectively. The correct insertion was verified by PCR and DNA

sequencing (Applied Biosystems, Foster City, USA). The resulting plasmids were transferred into *B. cenocepacia* H111 by triparental mating (de Lorenzo and Timmis, 1994).

Bioassays for the detection of DSF family signals

Cross-streak experiments: Both test and sensor strains were streaked in 90° with ca. 5 mm distance on an LB agar plate. Plates were incubated overnight at 37 °C (for luminescence detection) and then over night at room temperature (for fluorescence detection). As substrate for the luciferase, 10 µl Decanal (Sigma) was added to the lid of the plate. Luminescence and fluorescence were detected using a NightOWL LB 983 (Berthold Technologies). The background level was adjusted to the negative control (H111ΔcepI rpfF_{Bc}) or the auto fluorescence of the test strains. For *BDSF quantification in liquid cultures* the sensor strain was inoculated in LB (1:50, kanamycin 100 µg/ml) and grown to an OD_{600} of 1.8 - 2.0. The culture was diluted 1:1 in LB or with the supernatant of a strain to be tested for BDSF synthesis. Synthetic molecules to be tested for activity were directly mixed with the in LB diluted biosensor strain (10 µM unless otherwise stated). From this bacterial culture, 200 µl were added to 96 well plates (white, LumiNunc, VWR). The plates were incubated for 20 h at 30 °C. To detect activation of the biosensor by luminescence, 1 - 2 µl Decanal (Sigma) was added to each well. Relative luminescence units (RLU) were obtained using a plate reader (Synergy HT; Bio-Tek, Germany).

2.5.2 Figures S 2.1-S 2.5

Figure S 2.1

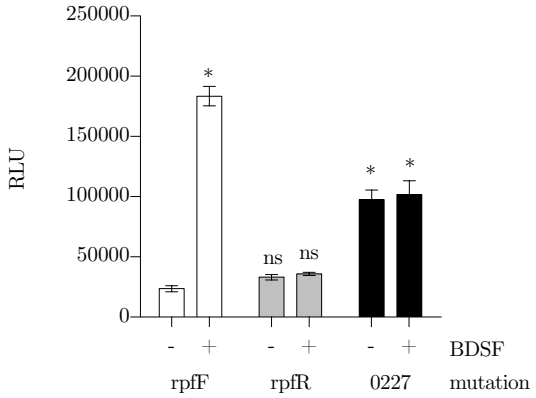

Figure S 2.1 The BDSF sensor RpfR but not BCAM0227 is required for *bclACB* promoter activity. The sensor plasmid pAN-L15 was introduced into the *rpfF*$_{Bc}$, the *rpfR* and the *BCAM0227* mutants and the recombinant strains were tested for biosensor activity in the presence (+) or absence (-) of 10 µM BDSF. white, H111-rpfF$_{Bc}$/pAN-L15; Grey, H111-rpfR/pAN-L15; Black, H111-bcam0227/pAN-L15. Error bars indicate SEM, n>2; * P<0.05; ns, not significant (ANOVA, oneway, Bonferroni post-test with 95 % CI). RLU, relative light units.

Figure S 2.2

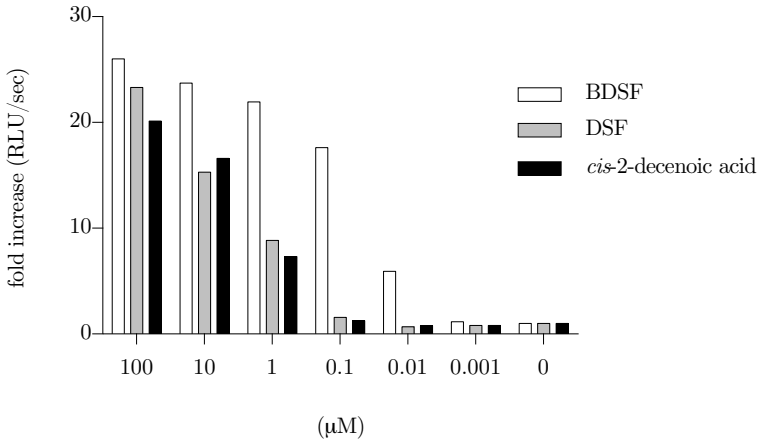

Figure S 2.2 Specificity and sensitivity of the H111-rpfF$_{Bc}$/pAN-L15 biosensor. Induction of bioluminescence was measured after the addition of molecules structurally related to BDSF in concentrations ranging from 0.001 µM to 100 µM. Fold increase is relative to the bioluminescence of the control without BDSF.

Figure S 2.3

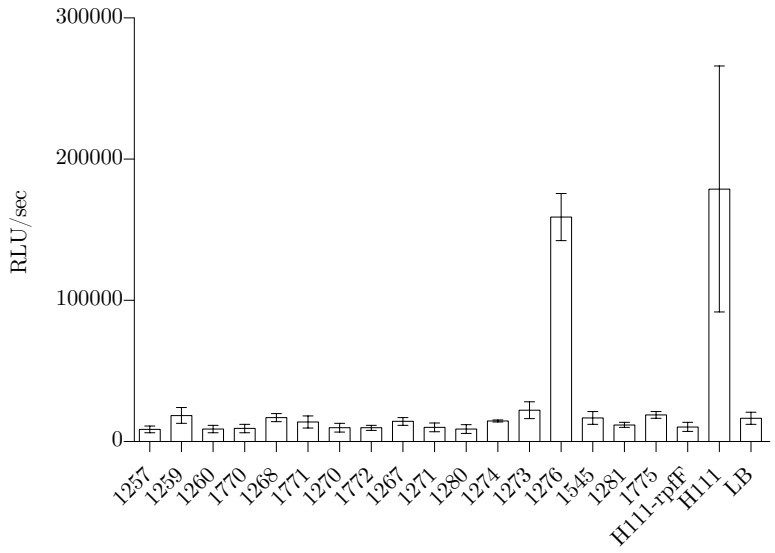

Figure S 2.3 *Burkholderia* strains tested for the production of DSF family signal molecules in liquid culture bioassays. Supernatants of overnight cultures of various *Burkholderia* strains (names are according to Table S 2.1) were tested for the induction of the biosensor *B. cenocepacia* H111-rpfF/pAN-L15. Error bars indicate SEM, n=2.

Figure S 2.4

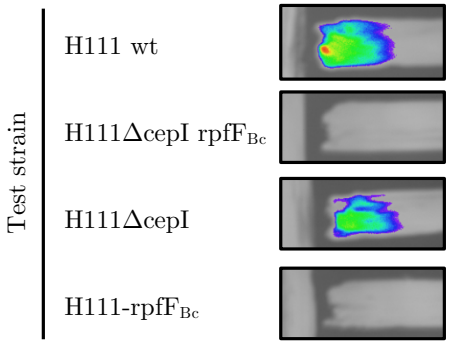

Figure S 2.4 The *gfp*-based biosensor is induced by BDSF-synthesizing strains. The sensor strain *B. cenocepacia* H111-rpfF/pAN-G3 (horizontal) was tested for induction by test strains (vertical) in cross-streak experiments. Only BDSF producers (*B. cenocepacia* H111 wt and H111ΔcepI) induce reporter gene expression. Background level was reduced to the autofluorescence level of the strains (1000 cts).

Figure S 2.5

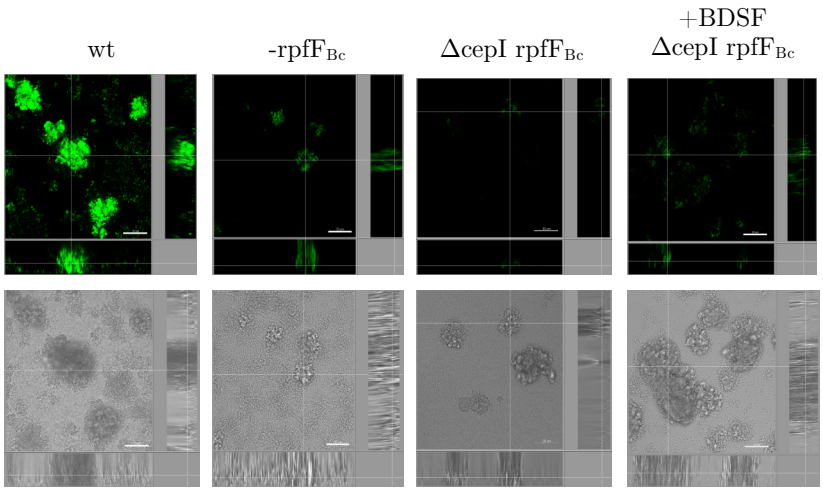

Figure S 2.5 The activity of the biosensor is strongly reduced in H111-rpfF$_{Bc}$ and H111ΔcepI rpfF$_{Bc}$ biofilms when compared to biofilms formed by the wild type strain. Biofilms were grown in flow-through cells as described previously (Inhülsen *et al.*, 2012). A 48 h old biofilm of H111ΔcepI rpfF$_{Bc}$ was supplemented with 10 µM BDSF for 3 h. Biofilms were analysed after 72 h of incubation using a confocal laser scanning microscope (Leica, DM 5500 Q, Wetzlar, Germany). Green fluorescence (upper panel) is indicative of BDSF production. BF, bright field (lower panel); scale bar: 30 µm.

Figure S 2.6

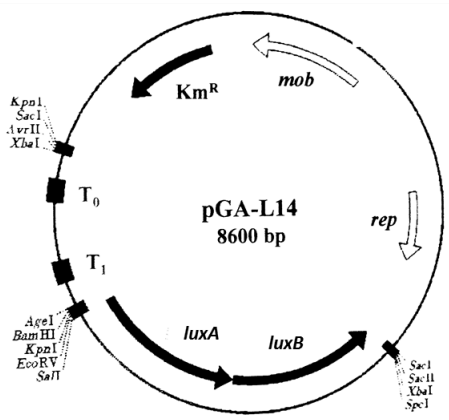

Figure S 2.6 Vector Map of the plasmid pGA-L14. The *cis*-2 fatty acid specific promoter region was cloned into the BamHI restriction site of pGA-L14. Figure taken from (Geiselhöringer, 1999), unpublished.

2.5.3 Tables S 2.1 - S2.2

Table S 2.1 *Burkholderia* strains tested in cross-streak experiments for induction of the *luxAB*-based biosensor H111-rpfF/pAN-L15 and presence of RpfR and $RpfF_{Bc}$ orthologs in sequenced *Burkholderia* species.

Group[a]	Species	Strain		ID[a]	Reaction[b]	RpfF homologue Identity (%)[c]	RpfF homologue Accession No.	RpfR homologue Identity (%)[c]	RpfR homologue Accession No.
Bcc	*B. ambifaria*	AMMD	LMG19182	883	++	94.43	Bamb_5286	91.3	Bamb_5285
	B. anthina		LMG20983	885	++	n.a.	n.a.	n.a.	n.a.
	B. arboris		LMG24066	1688	++	n.a.	n.a.	n.a.	n.a.
	B. cenocepacia	J2315	LMG16656	1074	++	100	BCAM0581	99.85	BCAM0580
		H111			++		I35_4475		I35_4474
	B. cepacia		LMG18821	604	++	100	NP88_RS13490	99.85	NP88_RS13495
	B. contaminans		LMG23361	1689	++	n.a.	n.a.	n.a.	n.a.
	B. diffusa		LMG24065	1690	++	n.a.	n.a.	n.a.	n.a.
	B. dolosa		LMG18943	639	++	96.86	BDSB_RS23770	92.35	BDSB_RS23765
	B. lata		LMG6993	99	++	94.77	Bcep18194_B2572	96.1	Bcep18194_B2573
	B. latens		LMG24064	1692	++	n.a.	n.a.	n.a.	n.a.
	B. metallica		LMG24068	1693	++	n.a.	n.a.	n.a.	n.a.
	B. multivorans		LMG18825	571	++	94.77	BURMUCF1_RS18925	92.8	BURMUCF1_RS18930
			LMG16660	572	++	94.43	Bmul_5121	92.8	Bmul_5122
	B. pyrrocinia		LMG14191	891	++	95.82	JM78_RS17730	95.95	JM78_RS17725
	B. seminalis		LMG24067	1694	++	n.a.	n.a.	n.a.	n.a.
	B. stabilis		LMG14291	613	++	n.a.	n.a.	n.a.	n.a.
	B. ubonensis		LMG24263	1571	++	89.9	BUBO0001_RS32575	86.21	BUBO0001_RS32570
	B. vietnamiensis		LMG18835	575	++	94.77	Bcep1808_4033	91	Bcep1808_4032
PBE	*B. caledonica*		LMG19076	1259	+	70.88	BCA01S_RS20415	66.42	BCA01S_RS20420
	B. caribensis		LMG18531	1260	-	72.98	K788_RS04930	65.17	K788_RS04925
	B. fungorum		LMG16225	1770	-	71.23	BFU01S_RS04975	65.57	BFU01S_RS04970
	B. graminis		LMG18924	1268	+	71.58	BGRAMDRAFT_RS01885	65.87	BGRAMDRAFT_RS01890
	B. ginsengisoli		LMG24044	1771	-	71.58	BGI01S_RS10905	65.32	BGI01S_RS10910
	B. kururiensis		LMG19447	1270	-	71.93	G118_RS0118415	65.77	G118_RS0118425
	B. tropica		LMG22274	1280	-	n.a.	n.a.	n.a.	n.a.
	B. phenazinium		LMG2247	1271	-	n.a.	n.a.	n.a.	n.a.
	B. phenoliruptrix		LMG22037	1775	+	72.63	BPAC_RS10170	66.22	BPAC_RS10165
	B. phymatum	STM815	LMG21445	890	-	74.39	Bphy_5278	66.07	Bphy_5279
	B. phytofirmans	PsJN	LMG22487	1273	-	71.58	Bphyt_5455	66.17	Bphyt_5454
	B. sordidicola		LMG22029	1276	++	43.96	FH08_RS0111830	41.31	FH08_RS0112455
	B. terricola		LMG20594	1545	+	n.a.	n.a.	n.a.	n.a.

	B. xenovorans	LMG21463	1281	-	71.23	DR64_RS38385	66.27	DR64_RS38390
PP	*B. andropogonis*	LMG2129	1257	-	n.a.	n.a.	40.65	W813_RS30570
	B. glumae	LMG2196	1342	-	36.84	NCPPB3923_RS18065	43.11	NCPPB3923_RS17540
	B. plantarii	LMG9035	1274	+	n.a.	n.a.	n.a.	n.a.

a. Group: Bcc; plant beneficial environmental strains (PBE); plant pathogens (PP); ID: according to our collection.

b. Reaction: assessed by cross-streak experiments as described in experimental procedures. The assay was repeated twice. Background cts was determined by streaking the sensor next to H111-rpfF. +, weak induction (<4 fold vs. background cts); ++, strong induction (>4 fold vs. background cts); -, no signal above background.

c. RpfFR homologue Identity according to online blast search (http://beta.burkholderia.com/); n.a., not applicable.

Table S 2.2 Bacterial strains and plasmids used in this study.

Strain or Plasmid	Phenotypes and/or characteristics	Reference or source
Escherichia coli		
DH5α	F⁻ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *recA1 endA gyrA96 thi-1 hsdR17 supE44 relAI deoR*(*U169*)	(Hanahan, 1983) Invitrogen, Laboratory collection
HB101 /pRK600	F⁻ *supE44 hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY2 galK2 rpsL20 xyl-5 mtl-1 recA thi pro leu hsdR⁻ M⁺* Sm^r; RK2-*mob*⁺ RK2-*tra*⁺, *ori* ColE1; Cm^r	(Boyer and Roulland-Dussoix, 1969; Kessler *et al.*, 1992)
Burkholderia cenocepacia		
H111	CF isolate from Germany	(Huber *et al.*, 2001; Carlier *et al.*, 2014)
H111-rpfF_{Bc}	*rpf*F_{Bc} mutant of H111, Cm^R	(Schmid *et al.*, 2012)
H111 ΔcepI rpfF_{Bc}	*cepI* rpfF_{Bc} double mutant, Cm^R	(Schmid *et al.*, 2012)
H111ΔcepI	*cepI* mutant of H111, markerless	(Schmid *et al.*, 2012)
H111-rpfR	*rpfR* mutant of H111, Gm^R	(Deng *et al.*, 2012)
H111-BCAM0227	*Bcam0227* mutant of H111, Gm^R	(Deng *et al.*, 2012)
Plasmids		
pGA-G2	Broad host-range *gfp*mut3*-based promoter probe vector, Gm^R	Laboratory collection
pGA-L14	Broad host-range *luxAB*-based promoter probe vector, Km^R	Laboratory collection
pAN-L15	pGA-L14 containing the *bclA* promoter region, Km^R	This study
pAN-G3	pGA-G2 containing the *bclA* promoter region, Gm^R	This study

Chapter 3

Interplay of the AHL- and BDSF-based QS systems for expression of the lectin operon *bclACB* in *B. cenocepacia* H111

3.1 Introduction

Lectins are glycan-binding proteins that are expressed in all domains of life and are involved in a variety of biological processes, including defence mechanisms, cell adhesion or host-microbe interactions (Peumans and Van Damme, 1995; Collins and Paulson, 2004; Imberty and Varrot, 2008).[2] For example, lectins of pathogenic microorganisms are often required for recognition and adhesion to a host by targeting specific glycoconjugates at cell surfaces. The opportunistic human pathogen *Burkholderia cenocepacia* H111 produces at least three lectins; BclA (I35_4184), BclC (I35_4183) and BclB (I35_4182). All three lectins have a mannose-binding C-terminal domain, that has sequence similarity to PA-IIL (LecB) of *Pseudomonas aeruginosa* (Lameignere *et al.*, 2008).[3] The lectins BclC and BclB contain additional N-terminal domains. The N-terminal domain of BclC binds L-fucose and elicits a carbohydrate-independent immunostimulatory activity on epithelial cells, suggesting a role in pathogenicity (Sulák *et al.*, 2010; Sulák *et al.*, 2011). In contrast, the N-terminal domain of BclB is still uncharacterized and shows no similarity to any other known protein domain.

The nematode *Caenorhabditis elegans* is often used as a model to study infection processes mediated by *B. cenocepacia* (Huber *et al.*, 2004; Schwager *et al.*, 2012). Recently, the individual contribution of the *B. cenocepacia* H111 lectins BclA, BclB and BclC to pathogenicity was analysed in a *C. elegans* infection model (Wohlschlager, 2013). When expressed in *Escherichia coli,* each of the three lectins was found to be toxic to *C. elegans pmk-1*(*km-25*). However, upon testing purified BclA and BclB (0.5 mg/ml) in *C. elegans* assays, no toxicity was observed for BclA, whereas BclB was toxic. Using truncated variants of the protein, it was shown that toxicity of BclB to *C. elegans* does not depend on its mannose-binding C-terminal domain but on its N-terminal domain, which binds to the intestine of *C. elegans*. However, glycan array analysis (glycan array 2058, http://www.functionalglycomics.org) of the BclB N-terminal domain did not reveal binding to mammalian type glycans (A. Imberty, personal communication).

In addition to multiple other virulence genes, expression of the lectin operon *bclACB* was found to be QS regulated by both AHL- and BDSF-based signalling systems in *B. cenocepacia* H111 (Inhülsen *et al.*, 2012; Schmid *et al.*, 2012). Initially, the operon was shown

[2] Additional information on lectins is available in Section 1.4.1.
[3] Additional information on lectins in *B. cenocepacia* H111 is available in Section 1.4.2.

to be regulated by the AHL-receptor CepR (Inhülsen *et al.*, 2012).[4] A more recent study has analysed the BDSF-dependent regulon and revealed its involvement in the regulation of *bclACB* (Schmid *et al.*, 2012). By studying the overlap of the BDSF- and the AHL-dependent regulon, persuasive results were presented that expression of *bclACB* primarily depends on BDSF-dependent activation, but the CepIR system synergistically enhances its expression (Schmid *et al.*, 2012). The AHL- and BDSF-dependent QS systems regulate a specific as well as an overlapping set of genes (Schmid *et al.*, 2012). It has been hypothesized, that a BDSF-dependent c-di GMP effector either controls gene expression in parallel to the AHL-based QS system, or the two QS systems converge to regulate a common downstream regulator, which then regulates expression of target genes.

In order to understand how the AHL- and BDSF-dependent QS system jointly control gene expression, the promoter region of the lectin operon *bclACB* was studied in detail. Analysis of wild-type or mutated promoter regions provided evidence that both the AHL- and BDSF-dependent QS systems regulate gene expression at positions in the *bclA* promoter region, probably at both the transcriptional and posttranscriptional levels. In addition, we show that the N-terminal domain of lectin BclB is toxic to nematodes.

3.2 Results

3.2.1 The N-terminal part of BclB is required for nematotoxicity by binding to an unknown target

In order to characterize the ligand of the BclB N-terminal domain, we tested six *C. elegans* strains (kindly provided by Dr. Markus Künzler, ETH Zurich) with defined mutations in genes required for carbohydrate modification of proteins or lipids, for resistance towards *E. coli* BL21(DE3) producing BclB or the BclB N-terminal domain. The *C. elegans* mutants used were *bre-5*, *aman-2*(*tm1078*), and the double mutant *hex-3*(*tm2725*);*hex-2*(*tm2530*), which were compared to their parent strain *C. elegans* N2, and *samt-1*(*op532*), *ger-1*(*op499*), and *galt-1*(*op497*), which were compared to their parent strain *C. elegans pmk-1*(*km25*). Toxicity analysis was performed as previously described, by scoring development of *C. elegans* L1 larvae, after 48 hours of feeding with *E. coli* BL21(DE3) expressing the protein of interest (Künzler *et al.*, 2010). Results indicated that none of the *C. elegans* glycan mutants showed an attenuated

[4] Additional information on QS in *Burkholderia* is available in Section 1.3.

phenotype (Figure 3.1). All the *C. elegans* were affected similarly to their parent strain, and did not develop when fed with the *E. coli* strains producing BclB or its N-terminal domain.

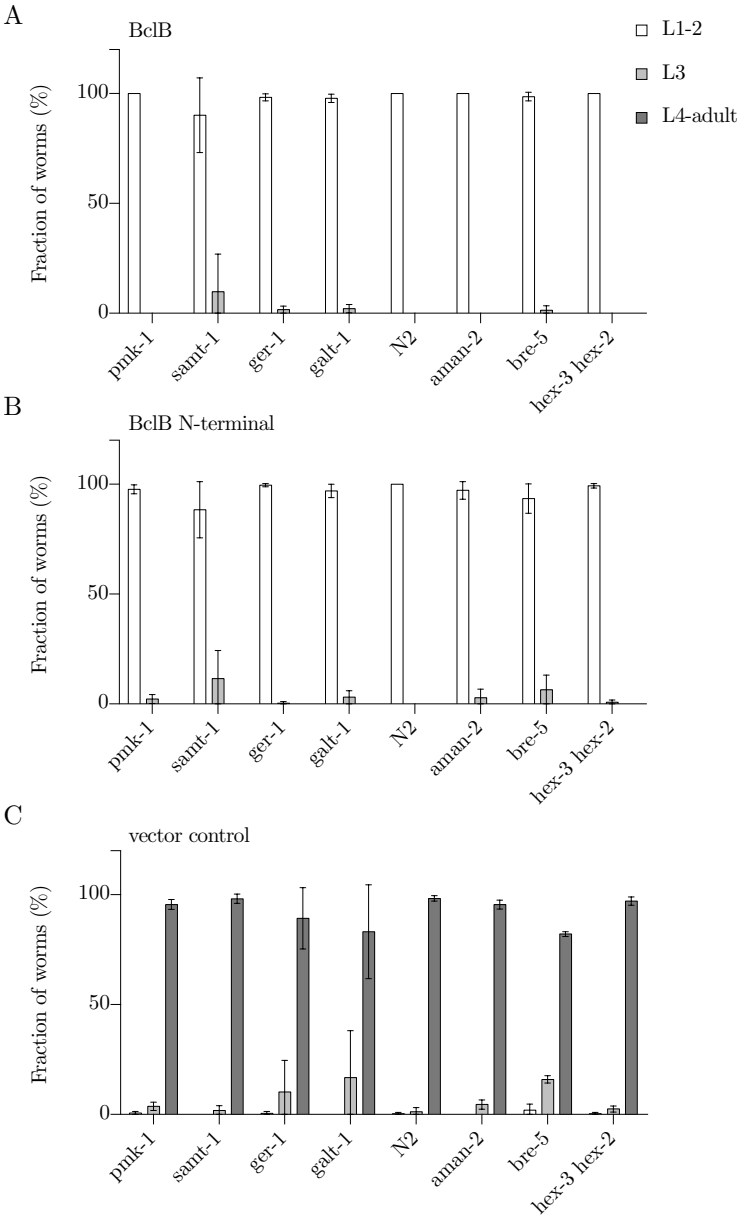

Figure 3.1 The recombinant BclB and the BclB N-terminal part display toxicity towards *C. elegans* glycan mutants. *E. coli* BL21 (DE3) cells expressing BclB (**A**), the N-terminal domain of BclB (**B**) or containing an empty vector (**C**) were fed to *C. elegans* N2 or *pmk- 1*(*km25*) and isogenic mutants. *C. elegans* development from L1-2 (white), L3 (light grey) to L4-adult (dark grey) was analysed as described in Section 3.5, Material and Methods. Bars represent the average of three biological replicates. Error bars indicate S.D.

3.2.2 Expression of the lectin operon is medium-dependent

In order to study environmental conditions promoting expression of the lectin operon in *B. cenocepacia* H111, a chromosomal *bclA-lacZ*-fusion was constructed. An in-frame translational fusion was constructed, in which the coding sequence of *bclA* downstream of the start codon was replaced by *lacZ*, generating *B. cenocepacia* H111 *bclA*::*lacZ*. This chromosomal fusion allowed expression of *lacZ* under control of the native *bclA* promoter. Bacterial cells were spot inoculated on a range of different agar plates (LB, NB, AB-Citrate, AB-Mannose, AB-Mannitol, PIA) supplemented with X-gal (50 µg/ml). After incubation for 48 h at 37 °C, followed by incubation at room temperature for two to four days, the bacterial macrocolonies were visually inspected for β-galactosidase activity, as evidenced by depth of blue colouration. The *bclA* promoter was active on all media tested (Figure 3.2). However, the most intense blue colouration, indicative of a high level of *lacZ* expression, was observed on NB, AB-Citrate and AB-Mannose plates. On these plates, an intensely blue ring was evident surrounding the centre of the macrocolony, with a lighter shade of blue in the centre and edge of the macrocolony. On AB-Mannose and AB-Mannitol plates, *bclA-lacZ* expression was strongest in the cells close to the agar surface of the macrocolony.

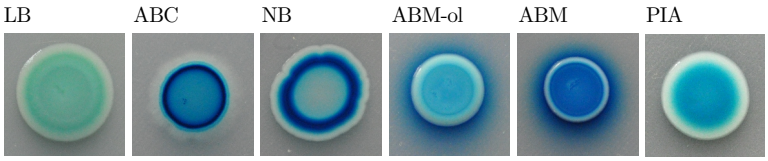

Figure 3.2 The expression of the lectin operon *bclACB* is medium-dependent. *B. cenocepacia* H111 derivative with a chromosomal *bclA-lacZ* fusion was spot inoculated on solid agar media plates supplemented with 50 µg/ml X-gal. LB, Luria-Bertani; ABC, AB-Citrate; NB, nutrient broth; ABM-ol, AB-Mannitol; ABM, AB-Mannose; PIA, *Pseudomonas* isolation agar.

3.2.3 *In silico* analysis of the *bclACB* promoter region

The lectin operon *bclACB* (I35_4184-82) is located on chromosome 2 in *B. cenocepacia* H111. There is an intergenic region of 941 bp between *bclA* and the inversely transcribed gene I35_4185 (encoding a putative 2-isopropylmalate synthase). Upstream of I35_4185 is *cepR2* (I35_4186), which codes for an orphan LuxR homolog (Malott *et al.*, 2009). The chromosomal organisation of this region is depicted in Figure 3.3A.

In previous studies, the AHL-dependent expression of *bclACB* was analysed using a plasmid-based transcriptional fusion of 638 bp from the *bclACB* promoter region to *lacZ* (Inhülsen *et al.*, 2012). In order to identify potential binding sequences within the promoter region, we performed an *in silico* analysis. Putative -35 and -10 *E. coli* consensus sequences were identified 124 bp and 104 bp upstream of the *bclA* start codon, respectively. Analysis of our previous RNA-Seq data (Lardi *et al.*, 2015) revealed a transcriptional start site (TSS) at position -90 bp (relative to the *bclA* transcriptional start codon), which is in good agreement with the position of the identified consensus sequences. We also identified several inverted repeats of up to 12 bp long in the intergenic region, and a direct repeat/ palindrome. These are depicted in Figure 3.3B. The presence of various repeats in the 5' untranslated region (5'-UTR) of the *bclACB* mRNA could potentially lead to RNA folding (ΔG= -39.10 kcal/mol using the mfold web server) as shown in Figure 3.3C (Zuker, 2003). It is worth noting that in the secondary structure prediction shown, the translation initiation ATG codon is hidden within a stem-loop structure and would not be available for translation initiation.

In *P. aeruginosa*, the FNR-type regulator ANR regulates the expression of target genes under oxygen limited conditions (Winteler and Haas, 1996). Recently, Pessi *et al.* showed that expression of both the FNR/ANR-type ortholog BCAM0049 and the lectin *bclC* is increased under micro-oxic conditions (Pessi *et al.*, 2013). In agreement with this finding, a putative ANR binding sequence (*anr* box, ATG-N8-CAA) was identified 515 bp upstream of the predicted TSS of *bclA*, which may be important for lectin biosynthesis under low oxygen conditions.

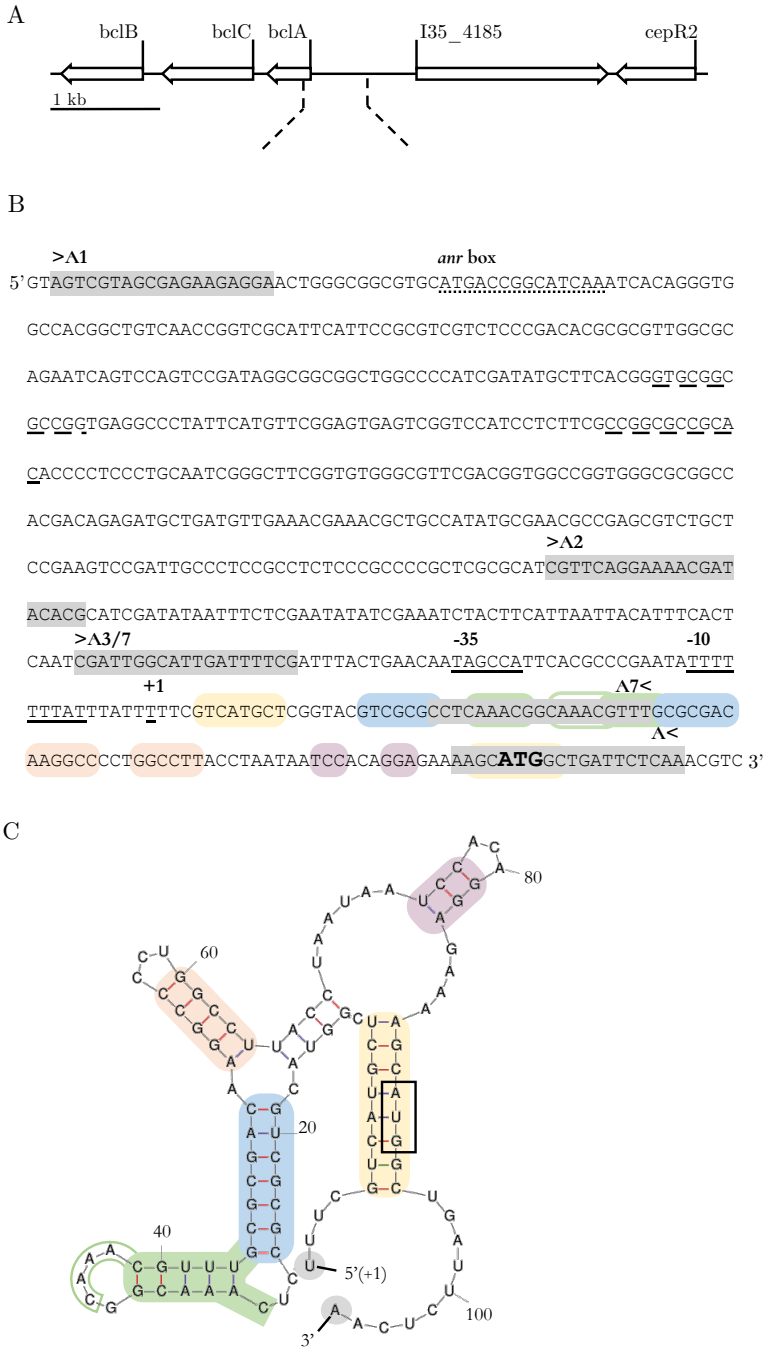

Figure 3.3 Genomic organization of the lectins *bclACB* on chromosome 2 in *B cenocepacia* H111, **A**. Arrows indicate the direction of transcription for each ORF. Annotations refer to the *B. cenocepacia* H111 genome (Carlier *et al.*, 2014). Dotted lines indicate the promoter region analysed in this study. **B**, Nucleotide sequence of the lectin *bclACB* promoter region. Characteristics and primer binding sites are indicated as follows: start codon of *bclA*, bold; primer binding sites, grey; TSS +1, underlined; putative -10 and -35 regions, underlined; *anr* box, dotted underlined; 12 bp inverted repeat, dashed underlined. In colour are the repeats shown that were identified downstream of the TSS. **C**, Predicted RNA secondary structure of the *bclA* 5' untranslated leader region. The *bclA* start codon AUG is boxed. The coloured, labelled repeats correspond to the labelled repeats in B.

3.2.4 BDSF controls *bclACB* expression via the 5'-UTR

Transcriptional promoter fusions were constructed by cloning different portions of the *bclACB* promoter region in front of the *lacZ* reporter gene in the promoter probe vector pSU11Tp, giving rise to pSU-A1 (653 bp), pSU-A2 (251 bp), pSU-A3 (171 bp), pSU-A4 (501 bp) and pSU-A7 (109 bp) as shown in Figure 3.4A. The constructs were introduced into the QS double mutant *B. cenocepacia* H111ΔcepI rpfF$_{Bc}$ and β-galactosidase activities were measured after addition of 10 μM BDSF and/or 200 nM AHL. The addition of BDSF resulted in a similar fold change in activity for strains harbouring pSU-A1, pSU-A2 or pSU-A3 (approximately 3-fold induction), while no increase was observed for plasmids pSU-A4 and pSU-A7 (Figure 3.4B, C). The promoter regions present in pSU-A3 and pSU-A7 consisted of the DNA from +90 to -66 relative to TSS and of +43 to -66, respectively (Figure 3.4A). Therefore, the data suggest that the full 5'-UTR of *bclA*, present in pSU-A3, is required for BDSF-dependent regulation of the lectin operon *bclACB*.

The highest β-galactosidase activity was observed when pSU-A7 was present. The 66 bp DNA fragment present in this plasmid contains the predicted -35 and -10 promoter regions and the 5'-UTR from position +1 to +43, relative to TSS. Expression of *lacZ* from this plasmid was found to be independent of BDSF (Figure 3.4B), and was significantly elevated compared to that from the construct containing the entire promoter region (pSU-A1). These data suggest that a negative control element is located in the 5'-UTR and that downregulation is relieved in the presence of BDSF.

Activity in the presence of pSU-A2, pSU-A3, pSU-A4 and pSU-A7 was unaffected by addition of AHL to the growth medium (Figure 3.4C). However, upon addition of both AHL (200 nM) and BDSF (10 μM) promoter activity was increased from plasmid pSU-A1, but not from pSU-A2 or pSU-A3. This suggests that i) the region between nucleotides -146 to +90 is not required for AHL-dependent activation and ii) the promoter is only regulated in response to AHLs in the presence of BDSF.

The *bclA* promoter fragment present in pSU-A4 was active in *B. cenocepacia* H111 grown in both LB and LB supplemented with BDSF, even though it lacked the predicted -35 and - 10 promoter regions (Figure 3.4B). This suggests that a second promoter might be present, upstream of the one we identified.

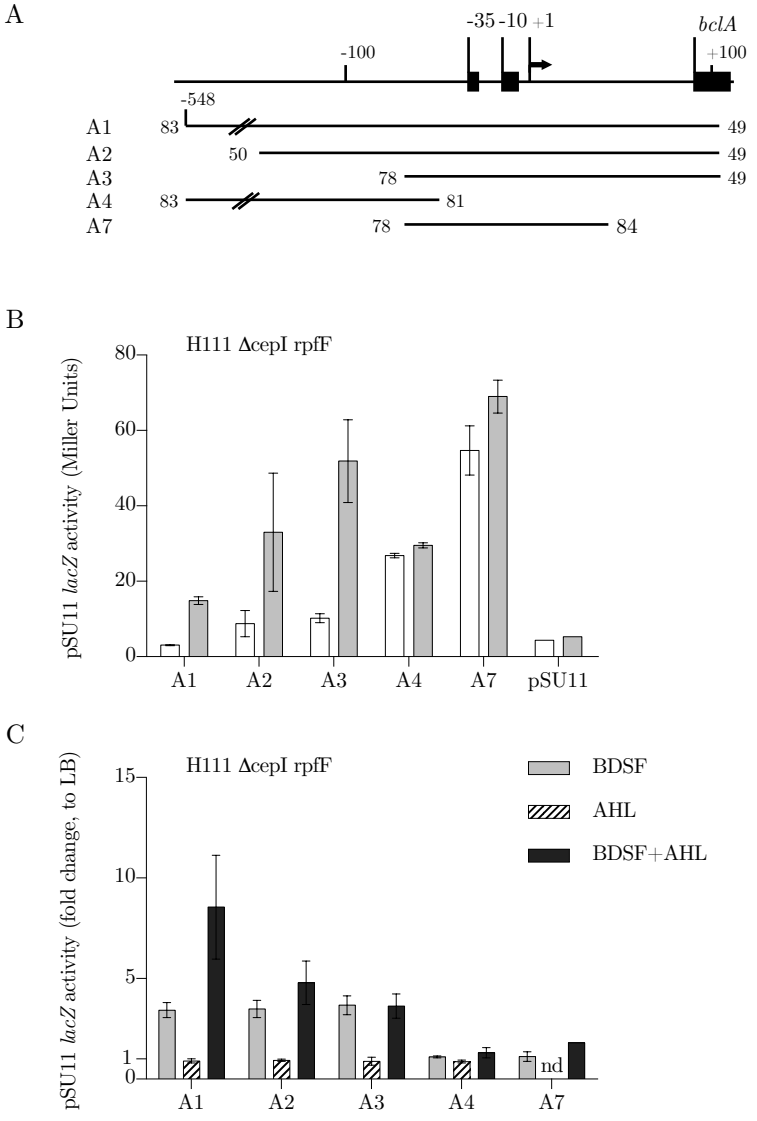

Figure 3.4 Analysis of *bclACB* promoter fragments activity. A, Schematic illustration of the promoter constructs generated in this study. The uppermost line depicts the *bclACB* promoter region, with -35 and -10 promoter regions and the TSS +1 indicated. The lower lines represent the promoter regions cloned into pSU11Tp, upstream of the reporter gene *lacZ*. Numbers indicate the primers used to generate the promoter fusion constructs. **B**, β-galactosidase activities of the QS double mutant H111 ΔcepI rpfF$_{Bc}$ carrying various plasmid-based transcriptional fusions (A1-A7). Culture samples were taken at the late exponential growth phase (OD_{600} = 2.3 - 2.8). Bacteria were grown in the absence (open bars) or presence (grey bars) of BDSF (10 μM). **C**, β-galactosidase activities in LB supplemented with either BDSF (grey bars), AHL (hatched bars) or BDSF and AHLs (black bars). Fold increase is relative to the value obtained for each construct in LB. Bars represent the average of three biological replicates. Error bars indicate SEM; nd, not determined.

To investigate whether the increase in activity in the presence of BDSF is due to relief of repression mediated via the promoter region or post-transcriptional regulation, we constructed a translational P*bclA-lacZ* fusion (pbdt-A3) containing the 156 bp promoter fragment from pSU11-A3. When this construct was introduced into the *rpfF* mutant, *lacZ* expression was 35-fold induced when the medium was supplemented with 10 μM BDSF (Figure 3.5).

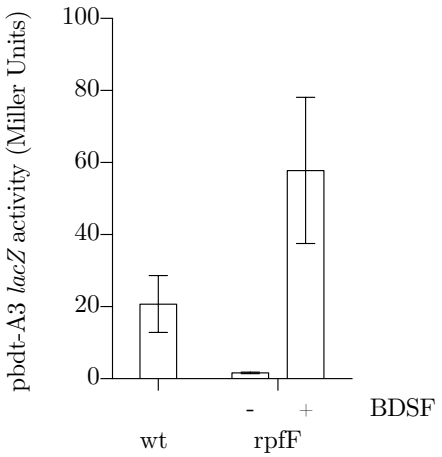

Figure 3.5 β-galactosidase activities of a translational P*bclA*3-*lacZ* fusion. Enzymatic activities of wildtype H111 and H111-rpfF containing the reporter plasmid pbdt-A3 were determined at an OD_{600} of 2.8. H111-rpfF was grown in the presence (+) or absence (-) of exogenously added BDSF (10 μM). Bars represent the average of four biological replicates. Error bars indicate SEM.

We also investigated expression of another BDSF-regulated gene; *hamA* (I35_4191, bcam0192 in J2315) (Schmid *et al.*, 2012; Jenul, 2016). The entire *hamA* promoter region was cloned as a 455 bp fragment into pSU11. We also constructed a reporter vector bearing a 246 bp DNA fragment containing the putative *hamA* -35 and -10 regions and 5' UTR, but lacking the region upstream of the promoter (Christian Jenul, laboratory collection). Both constructs were introduced into the *rpfF* mutant *B. cenocepacia* H111-rpfF, and reporter gene activities were determined in the presence and absence of BDSF. β-galactosidase activity was inducible on both plasmids by supplementation with BDSF (Figure 3.6). These results show that expression of *hamA* is also BDSF-regulated, mediated by either its promoter region or the 5'-UTR.

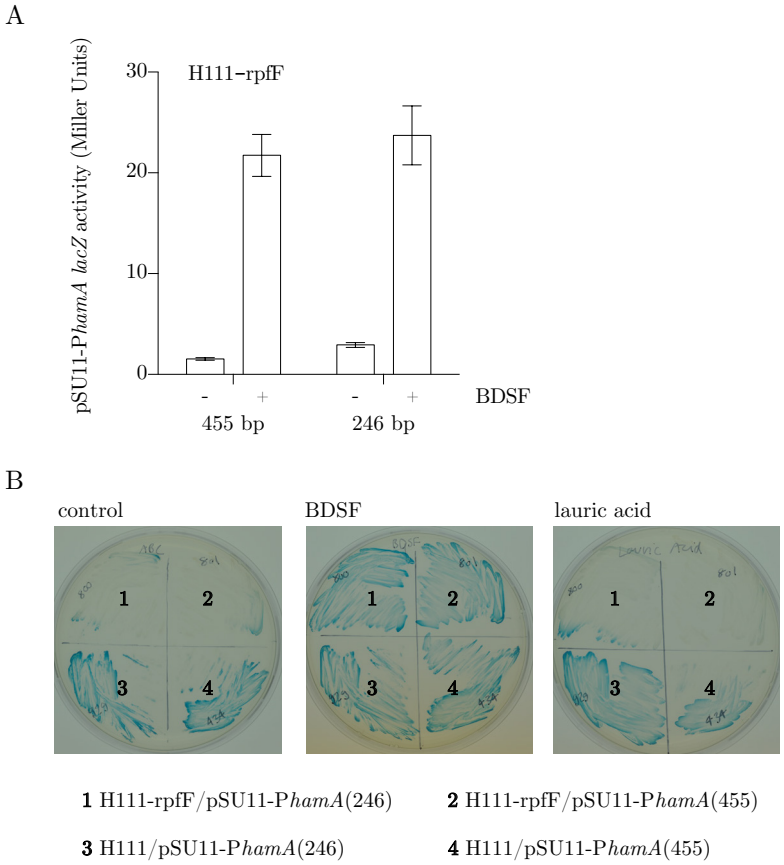

Figure 3.6 A 246 bp promoter fragment of *hamA* is sufficient for BDSF-dependent transcriptional control. A, A 455 bp and a 246 bp fragment of the *hamA* promoter were fused to *lacZ*. Reporter gene expression was analysed in the presence (+) or absence (-) of 10 μM BDSF in an *rpfF* mutant background. Bars represent the average of two biological replicates. Error bars indicate S.D. **B**, Reporter gene expression was analysed on AB-Citrate plates containing 10 μM BDSF or 10 μM lauric acid, the saturated isomer. Both fragments of the *hamA* promoter were active only in the presence of BDSF. The promoter fragments were active in the wild-type *B. cenocepacia* H111.

To investigate further, the native -10 and -35 consensus sequences of the lectin promoter in construct pbdt-A3 were replaced with an artificial *lac* promoter, while the native 5'-UTR was maintained, yielding plasmid pAS-1 (Figure 3.7). The construct was analysed in the *B. cenocepacia* wild-type H111, the *rpfF* mutant H111-rpfF and the double mutant H111ΔcepI rpfF_{Bc}. β-galactosidase activity was observed in the wild-type but not in the mutant backgrounds. However, in both mutants enzymatic activity was restored to wildtype level by exogenous addition of BDSF (Figure 3.7). These results provide further evidence that the 5'-UTR of the *bclACB* promoter region contains a negative regulatory element that is disarmed in a BDSF-dependent manner.

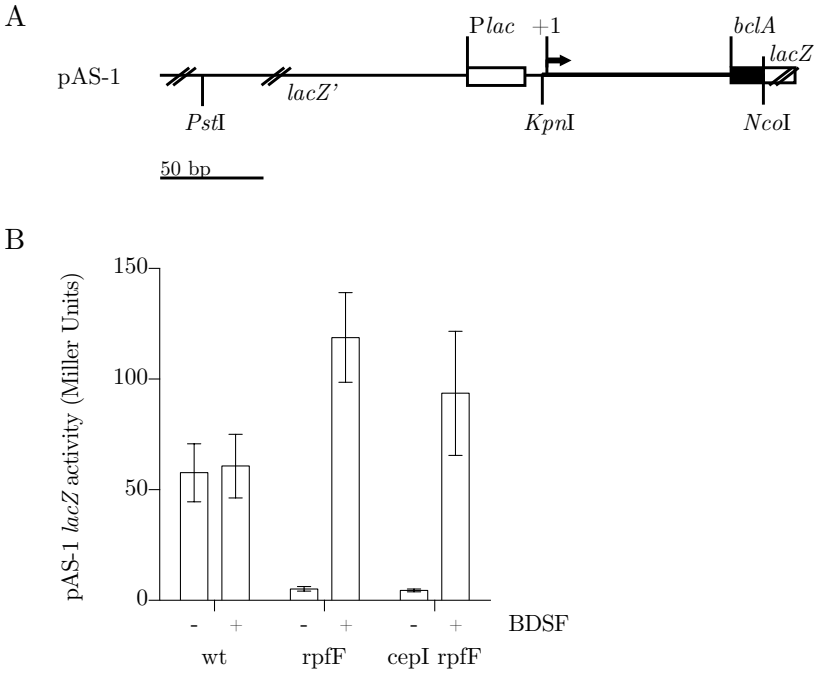

Figure 3.7 The 5'-UTR negatively regulates expression of *bclACB* in the absence of BDSF. **A**, schematic illustration of plasmid pAS-1. The *lacZ* promoter (P*lac*) was cloned upstream of the native 5'-UTR of a translational *bclA-lacZ* fusion. **B**, Plasmid pAS-1 was introduced into the H111 wildtype (wt) and mutants H111-rpfF and H111ΔcepI rpfF_{Bc}. Promoter activities were determined at an OD_{600} of 2.8. Cells were grown in the presence (+) and absence (-) of exogenously added BDSF (10 μM). Bars represent the average of three biological replicates. Error bars indicate SEM.

3.2.5 RpfR mediates BDSF-dependent regulation of the *bclACB* operon

To further analyse regulation of the *bclACB* operon, plasmid pAS-1 (Figure 3.7A) was introduced into various *B. cenocepacia* mutant backgrounds: H111-rpfR, H111-rpfFR, H111ΔcepI, H111ΔcepR, H111-rpoN and H111-1349 (bcam1349 in J2315, I35_5200 in H111, a c-di-GMP effector (Fazli *et al.*, 2011)). Measurement of β-galactosidase activities revealed that both the *rpfR* and *rpfFR* mutants showed reduced activity compared to the wildtype (Figure 3.8), which could not be rescued by the addition of BDSF. The finding that in the presence of BDSF reporter gene activity of pAS-1 was rescued in the *rpfF* but not the *rpfFR* mutant suggests that BDSF-dependent activation of gene expression is dependent on the BDSF receptor RpfR. The enzymatic activities of the *cepI*, *cepR*, *rpoN* or *bcam1349* mutants was unchanged relative to the wildtype. These results support a model in which the AHL- and BDSF-dependent QS systems regulate expression of the lectin operon via different *cis*-acting sequences in the 5' upstream regions of *bclACB*.

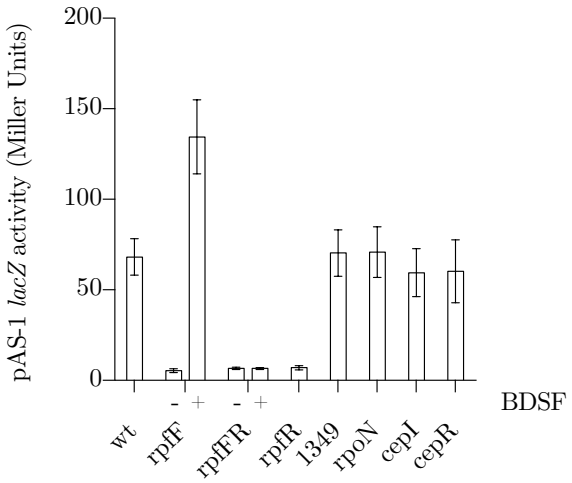

Figure 3.8 RpfR is involved in the BDSF-dependent regulation of *bclACB* expression. The vector pAS-1 was introduced into H111 wildtype and isogenic mutants as indicated. Reporter gene expression was measured at an OD_{600} of 2.5 in LB. For H111-rpfF and H111-rpfFR, promoter activity was measured in the absence (-) and presence of 10 μM BDSF (+). Bars represent the average of four biological replicates. Error bars indicate SEM.

3.3 Discussion

In general, pathogenic bacteria undergo drastic changes in growth conditions upon entering the human body or other hosts. The human body has an internal temperature of 37 °C and provides a humid environment. Depending on the location, a low pH (as found in macrophages), nutrient limitation and oxidative stress might exist (Cullen and McClean, 2015). Particularly in the thick mucus that lines the CF lung, bacteria encounter heterogeneous oxygen levels, different nutrient availabilities, various other organisms, antibiotic treatments and inflammatory responses (Yang *et al.*, 2011). Exposure to increased temperature, reduced oxygen concentration and organic nutrients have been shown to trigger expression of virulence factors (including the lectins *bclACB*) in *B. cenocepacia* J2315 (Sass *et al.*, 2013). Likewise, in *B. cenocepacia* H111, environmental stresses such as reduced oxygen- and nitrogen availability have been shown to alter gene expression, including expression of *bclACB* which was specifically up-regulated under both micro-oxic (0.5 % O_2) and nitrogen limited conditions (Pessi *et al.*, 2013; Lardi *et al.*, 2015). This doctoral study has expanded these investigations by studying expression of *bclACB* in *B. cenocepacia* H111 grown on different media. Macrocolony formation is an established biofilm model system that allows visualization of physiological differentiation in a spatial manner. The differentiation is driven by various gradients, including nutrients, oxygen, waste products, signalling molecules and stress conditions (Stewart and Franklin, 2008; Serra and Hengge, 2014).

Previous results have shown that in *B. cenocepacia* H111 the lectins BclACB are involved in biofilm formation (Inhülsen *et al.*, 2012) and may be required for cell-to-cell interactions (Marchetti *et al.*, 2012). In this study, we show that the lectin BclB exhibits significant nematotoxicity via its N-terminal domain, which was previously shown to bind to the intestine of the nematode *C. elegans* (Wohlschlager, 2013). However, despite several attempts, the target structure of the BclB N-terminal domain could not be identified ((Wohlschlager, 2013), A. Imberty, this study). This, in turn, raises the possibility that this domain is not a carbohydrate binding domain, but may instead bind a different class of molecules. The biological relevance and the preferred ligands of each of the three lectins are currently under investigation.

In this study, we provide evidence that the BDSF-based signalling system regulates expression of the lectin operon *bclACB* via the BDSF receptor RpfR and a *cis*-acting region within the 5'-UTR. In contrast, the AHL-based signalling pathway does not control gene expression via the 5'-UTR, but rather by affecting a secondary upstream promoter region.

This supports previous work that suggested that the AHL- and BDSF-based QS systems operate in parallel, rather than in a hierarchical fashion to control gene expression (Schmid *et al.*, 2012). However, the presence of an effector that merges the two pathways for controlling expression of a different subset of QS regulated genes cannot be excluded.

Our data suggest that expression of the *bclACB* operon is repressed via the 5'-UTR, but is de-repressed in the presence of BDSF. The mechanism might include an effector that alters mRNA conformation or stability (Rauhut and Klug, 1999; Hoe *et al.*, 2013), the nucleoid structure (Dillon and Dorman, 2010; Lee *et al.*, 2012), or it interacts with riboswitches (Smith *et al.*, 2011; Martinez and Vadyvaloo, 2014), or the effector acts as an anti-repressor by binding to the DNA. Negative regulators often bind to operators in the promoter region, such that they interfere with the binding of the RNA polymerase and thus repress transcription (Balleza *et al.*, 2009). A minority of repressor binding sites are also found downstream of the TSS (Madan Babu and Teichmann, 2003). Because the contribution of the BDSF-based system in the regulation of target genes is variable (Schmid *et al.*, 2012), it should also be considered, that transcription factors can have dual regulatory roles as activators and repressors. For example, the transcription factor FleQ binds to two sites in the promoter region of the *pel* operon in *P. aeruginosa*. One of these overlaps the TSS, and binding of FleQ here has been shown to repress gene expression. The other binding site is located upstream of the transcription start site, and when bound here FleQ activates gene expression in response to the c-di GMP level (Baraquet *et al.*, 2012; Baraquet and Harwood, 2016).

3.4 Supporting Information

3.4.1 Analysis of the *bclACB* leader region for putative binding sites

To identify potential binding sites for a regulator, or structural elements in the 5'-UTR the promoter fragment of pbdt-A3 was mutated by assembly PCR (Rouillard *et al.*, 2004). The mutated fragments (A-G, Figure S 3.1) were sequenced, ligated to pbdt-*lacZ* and introduced into *B. cenocepacia* H111 or H111 ΔcepI rpfF$_{Bc}$. Reporter gene expression of the strains was analysed on solid agar plates supplemented with X-Gal (50 µg/ml), and in liquid medium by taking β-galactosidase measurements. The majority of nucleotide substitutions did not significantly alter reporter gene expression compared to the wildtype (Figure S 3.2). However, the mutations present on fragment C1 abrogated activity in both the wild-type and the mutant backgrounds and were also not inducible by BDSF. The mutations introduced in fragment B resulted in increased activity in wildtype H111. Increased *lacZ* expression was also observed for fragments D8, E and F in the H111 ΔcepI rpfF$_{Bc}$ background. Our analysis identified certain nucleotides that might be relevant for regulation.

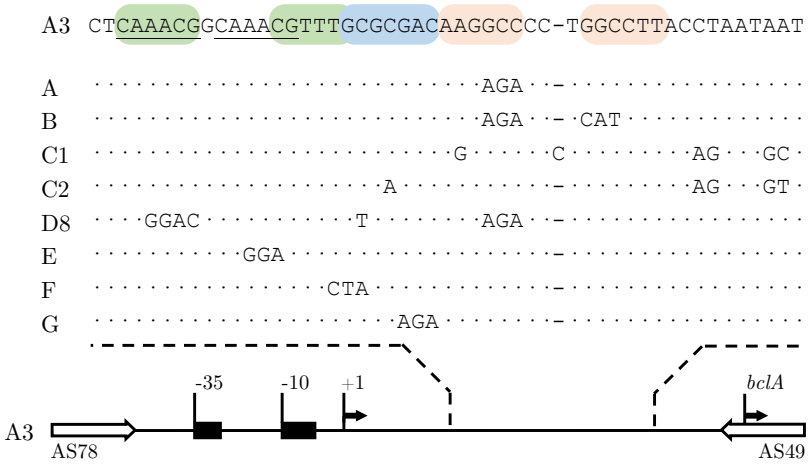

Figure S 3.1 Mutations introduced into the 5' leader region of *bclA*. The A3 fragment had the wild-type promoter sequence. The nucleotide sequence shows part of the native 5' leader sequence, containing a 6 bp direct repeat (underlined) as part of a larger palindromic sequence (CAAACG-N-CAAACGTTTG)), the repeats labelled in colour correspond those labelled in Figure 3.3. For each mutated fragment the nucleotide changes are shown. Open arrows indicate the two primers (AS78 and AS49) used to clone fragment A3 into the plasmid pbdt-*lacZ*.

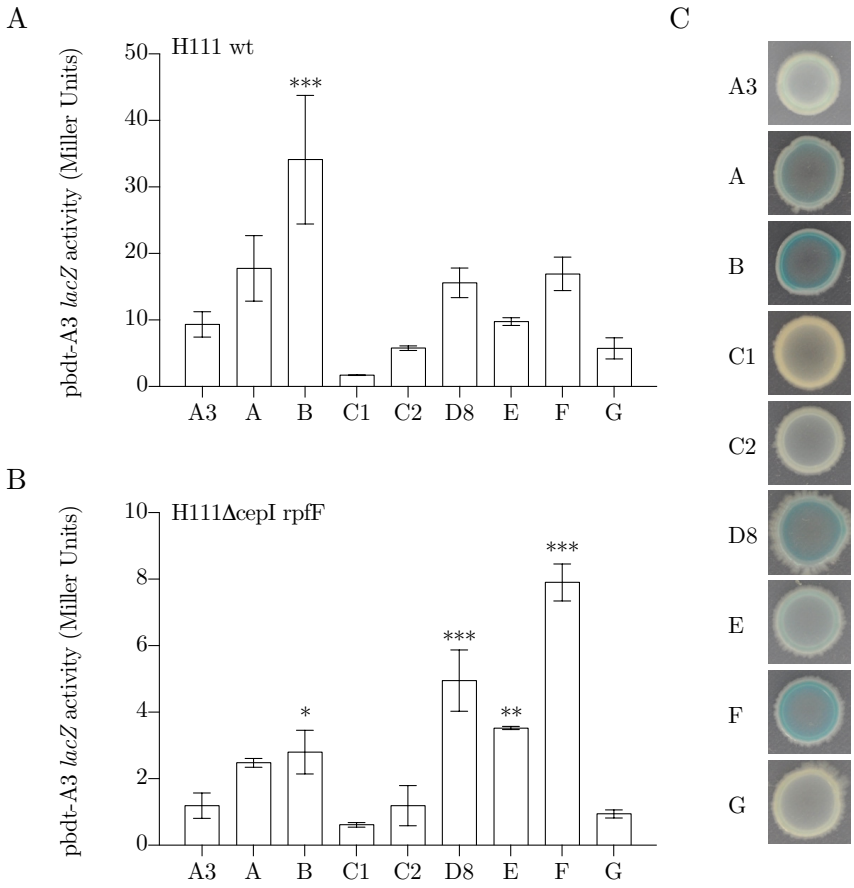

Figure S 3.2 Identification of nucleotides required for *bclACB* expression. Mutant variants (A-G) of the pbdt-A3 wild-type sequence were analysed for *lacZ* expression in the *B. cenocepacia* H111 wild type **(A)** and H111 ΔcepI rpfF$_{Bc}$ **(B)**. Bars represent the average of two biological replicates. Error bars indicate S.D. Two-way ANOVA, Dunnett's multiple comparisions test *vs*. A3, *** p value < 0.0005, ** p value < 0.005, * p value < 0.05. In **C**, *B. cenocepacia* H111 ΔcepI rpfF$_{Bc}$ carrying the mutated reporter plasmids were spot inoculated on AB-Citrate, X-Gal (50 μg/ml) plates, incubated for 48 h at 37 ºC. Blue colouration indicates *lacZ* expression.

3.5 Materials and Methods

Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3.1 and Table 3.2, respectively. *E. coli* and *B. cenocepacia* cells were routinely grown aerobically in Luria-Bertani (LB, Difco) at 37 ºC. Antibiotics were used at concentrations (μg/ml) as indicated in parentheses: for *E. coli*, ampicillin (100), kanamycin (25), gentamicin (10), chloramphenicol

(30), trimethoprim (50); and for *B. cenocepacia*, kanamycin (100), gentamicin (20), chloramphenicol (80) and trimethoprim (100).

Table 3.1 Bacterial strains used in this study.

Strains	Characteristics	Source/ Reference
Burkholderia cenocepacia		
H111	CF isolate from Germany, genomovar III	(Huber *et al.*, 2001; Carlier *et al.*, 2014)
H111 *bclA*::*lacZ*	chromosomal in-frame integration of *bclA* by *lacZ*	This study
H111-rpfF$_{Bc}$	*rpfF*$_{Bc}$::pSHAFT2 mutant of H111, Cm^R	(Schmid *et al.*, 2012)
H111-rpoN$_{Bc}$	*rpoN*$_{Bc}$::pSHAFT2 mutant of H111; Cm^R	(Lardi *et al.*, 2015)
H111ΔcepI	Δ*cepI deletion* mutant of H111, markerless	(Schmid *et al.*, 2012)
H111ΔcepI rpfF$_{Bc}$	Δ*cepI* and *rpfF*$_{Bc}$::pSHAFT double mutant, Cm^R	(Schmid *et al.*, 2012)
H111ΔcepR	*cepR*::Km mutant of H111, Km^R	(Huber *et al.*, 2003)
H111-rpfR	in-frame deletion *rpfR* mutant of H111, markerless	E. Steiner, laboratory collection
H111-rpfFR	in-frame double *rpfR* and *rpfF*mutant of H111, markerless	E. Steiner, laboratory collection
H111-1349	in-frame deletion I35_5200 (bcam1349 in J2315) mutant of H111, markerless	E. Steiner, laboratory collection
Escherichia coli		
DH5α	F$^-$ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *recA1 endA gyrA96 thi-1 hsdR17 supE44 relAl deoR(U169)*	(Hanahan, 1983)
BL21(DE3)	expression strain for BclB and BclB N-terminal	Novagen

Table 3.2 List of plasmids used in this study.

Plasmid	Characteristics	Source/ Reference
pBBR1MCS-5	Broad-host-range cloning vector, Gm^R	(Kovach *et al.*, 1995)
pSU11Tp	pSU11 derivative promoter probe vector, harboring dhfr cassette from pRN3, Tp^R	(Schmid *et al.*, 2012)
pSU-A1	pSU11Tp containing the predicted *bclA* promoter region, Tp^R	(Inhülsen *et al.*, 2012)
pSU-A2	pSU11Tp containing the predicted *bclA*2 promoter region, Tp^R	This study
pSU-A3	pSU11Tp containing the predicted *bclA*3 promoter region, Tp^R	This study
pSU-A4	pSU11Tp containing the predicted *bclA*4 promoter region, Tp^R	This study
pSU-A7	pSU11Tp containing the predicted *bclA*5 promoter region, Tp^R	This study
pSU11 P*hamA*-455	pSU11 containing the full *hamA* (I35_4191) promoter region of 455 bp, Gm^R	C. Jenul, laboratory collection
pSU11 P*hamA*-246	pSU11 containing the reduced *hamA* (I35_4191) promoter region, 246 bp, Gm^R	C. Jenul, laboratory collection
pbdt-*lacZ*	translational *lacZ* fusion vector, Gm^R	E. Steiner, laboratory collection
pbdt-A3	pbdt-*lacZ* containing the 172 bp (AS132-133) promoter region of *bclA*, Gm^R	This study
pAS-1	pbdt-*lacZ* containing an artificial lac promoter and the native 5'UTR of *bclA*, Gm^R	This study

pRK2013	Helper plasmid; RK2 derivative, *mob*$^{+}$ *tra*$^{+}$ *ori* ColE1; Km^{R}	(Figurski and Helinski, 1979)
pGEM T-easy	cloning vector for PCR products; Amp^{R}	Promega
pET-HisBclB	pET28a derivate carrying *bclB* for expression, Km^{R}	(Inhülsen *et al.*, 2012)
pDAIGm-SceI	Plasmid encoding the I-SceI nuclease gene, Gm^{R}	E. Steiner, laboratory collection
pGPI-SceI-MCS2-*lacZ*	pGPI-SceI carrying *lacZ*, Tp^{R}	E. Steiner, laboratory collection
pGPI-*lacZ*(109.110)	pGPI-SceI-MCS2-*lacZ* carrying the left homology arm of *bclA*, Tp^{R}	This study
pGPI-*lacZ*(109.110/111.112)	pGPI-SceI-MCS2-*lacZ* carrying the left and right homology arm of *bclA*, Tp^{R}	This study

Antibiotic-resistance of strains or plasmids: ampicillin (Amp^{R}), gentamicin (Gm^{R}), kanamycin (Km^{R}) and trimethoprim (Tp^{R}).

Oligonucleotides

Table 3.3 List of oligonucleotides (Microsynth AG, Balgach) used in this study.

Primer	**5'>3' Sequence**	**Application**
AS148_F	GCTCCTGCAGCCACGGTGTGCGTCCATG	to amplify Plac of pBBR1MCS-5 to create pAS-1
AS149_R	CTCGGGTACCCACACAACATACGAGC	to amplify Plac of pBBR1MCS-5 to create pAS-1
AS150_F	GCTCGGTACCTTTCGTCATGCTCGGTACG	to amplify 5'UTR of P*bclA* to create pAS-1
AS151_R	CTCGCCATGGTTTGAGAATCAGCCATGCTT	to amplify 5'UTR of P*bclA* to create pAS-1
AS132_F	GCGCGAATTCCGATTGGCATTGATTTTCG	to amplify P*bclA*3 (172bp) of H111 to create pbdt-A3
AS133_R	GCGCCCATGGTTTGAGAATCAGCCATGCTT	to amplify P*bclA*3 (172bp) of H111 to create pbdt-A3
AS79_F	GCCCTACACAAATTGGGAGA	pSU11
AS80_R	GACAGTATCGGCCTCAGGAA	pSU11
ES44_F	AGCTGATCCGGTGGATGAC	to test the plasmid pGPI-SceI used for in-frame deletions
ES45_R	ACGGTTGTGGACAACAAGC	to test the plasmid pGPI-SceI used for in-frame deletions
M13_F	TGTAAAACGACGGCCAG	
M13_R	CAGGAAACAGCTATGACC	
AS109_F	AGTCGAATTCGCGCAGAATCAGTCCAGTC	to generate chromosomal *lacZ*::*bclA* fusion
AS110_R	AGCTCTGCAGCAGCCATGCTTTTCTCCTGT	to generate chromosomal *lacZ*::*bclA* fusion
AS111_F	AGTCTCTAGAACGACAGCGACTACAACGAC	to generate chromosomal *lacZ*::*bclA* fusion
AS112_R	ACGTGGTACCCTTTTCCACTGGCCCTTCAC	to generate chromosomal *lacZ*::*bclA* fusion
P129_F	CAGTTGGTCTGGTGTC	to test chromosomal *lacZ*::*bclA* fusion
P130_R	TTCCCAGTCACGACG	to test chromosomal *lacZ*::*bclA* fusion
AS78_F	CTCGAGCGATTGGCATTGATTTTCG	to test chromosomal *lacZ*::*bclA* fusion
AS83_F	CTCGAGAGTCGTAGCGAGAAGAGGA	to amplify A1, A4 into pSU11, Tp^{R}
AS49_R	AAG CTT TTG AGAATCAGCCATGCTT	to amplify A1, A2, A3 into pSU11, Tp^{R}
AS50_F	CTC GAGCGTTCAGGAAAACGATACACG	to amplify A2 into pSU11, Tp^{R}
AS81_R	AAG CTTCGAAAATCAATGCCAATCG	to amplify A4 into pSU11, Tp^{R}
AS84_R	AAG CTTAAACGTTTGCCGTTTGAGG	to amplify A6, A7 into pSU11, Tp^{R}
AS78_F	CTCGAGCGATTGGCATTGATTTTCG	to amplify A3, A7 into pSU11, Tp^{R}
R15	GAATGGCTATTGTTCAGTAAATCGAAAATCAATGCC	to generate P*bclA*3 mutations in pbdt-*lacZ*
R51	GAGCATGACGAAAAATAAATAAAAAAATAT	to generate P*bclA*3 mutations in pbdt-*lacZ*

	TCGGGCGT	
R0	AATCGGAATTCGCGC	to generate P*bclA*3 mutations in pbdt-*lacZ*
F0	GCGCGAATTCCGATTGGCATTGATTTTCGATTT	to generate P*bclA*3 mutations in pbdt-*lacZ*
F33	ACTGAACAATAGCCATTCACGCCCGAATATTTTT	to generate P*bclA*3 mutations in pbdt-*lacZ*
F67	TTATTTATTTTTCGTCATGCTCGGTACGTCGCGCCT	to generate P*bclA*3 mutations in pbdt-*lacZ*
R89	ACGTTTGCCGTTTGAGGCGCGACGTACC	to generate P*bclA*3 mutations in pbdt-*lacZ*
F103	CAAACGGCAAACGTTTGCGCGACAAGAG	to generate P*bclA*3 mutations in pbdt-*lacZ*
R117	AGGTAAGGCCAGGTCTCTTGTCGCGCAA	to generate P*bclA*3 mutations in pbdt-*lacZ*
F131	ACCTGGCCTTACCTAATAATCCACAGGAGAAAA	to generate P*bclA*3 mutations in pbdt-*lacZ*
R145	GAGAATCAGCCATGCTTTTCTCCTGTGGATTATT	to generate P*bclA*3 mutations in pbdt-*lacZ*
F164	GCATGGCTGATTCTCAAACCATGGGCGCC	to generate P*bclA*3 mutations in pbdt-*lacZ*
R179	GGGAGCCATAGTTTCTGGCGCCCATGGTTT	to generate P*bclA*3 mutations in pbdt-*lacZ*
R193	AGAAACTATGGCTCCC	to generate P*bclA*3 mutations in *pbdt-lacZ*
R117b	AGGTAAGATGAGGTCTCTTGTCGCGCAA	to generate P*bclA*3 mutations in *pbdt-lacZ*
F131b	ACCTCATCTTACCTAATAATCCACAGGAGAAAA	to generate P*bclA*3 mutations in *pbdt-lacZ*
F103_c	CAAACGGCAAACGTTTGCGCGACAAGGC	to generate P*bclA*3 mutations in *pbdt-lacZ*
F131c	CCCTGGCCTTACAGAATGTTCCACAGGAGAAAA	to generate P*bclA*3 mutations in *pbdt-lacZ*
R117_c	CTGTAAGGCCAGGGGCCTTGTCGCGCAA	to generate P*bclA*3 mutations in *pbdt-lacZ*
R145_c	GAGAATCAGCCATGCTTTTCTCCTGTGGAACATT	to generate P*bclA*3 mutations in *pbdt-lacZ*
R15_51	GAGCATGACGAAAAATAAATAAAAAAATATTCGGGCGTGAATGGCTATTGTTCAGTAAATCGAAAATCAATGCC	to generate P*bclA*3 mutations in *pbdt-lacZ*
F103d2	CAGGACGCAAACGTGATCAAGACAAGGC	to generate P*bclA*3 mutations in *pbdt-lacZ*
F103d	CAAACGGCAAACGTGATCAAGACAAGGC	to generate P*bclA*3 mutations in *pbdt-lacZ*
R117d	AGGTAAGGCCAGGGGCCTTGTCTTGATC	to generate P*bclA*3 mutations in *pbdt-lacZ*
R89d2	ACGTTTGCGTCCTGAGGCGCGACGTACC	to generate P*bclA*3 mutations in *pbdt-lacZ*
F103_E	CAAACGGCAGGAGTTTGCGCGACAAGGC	to generate P*bclA*3 mutations in *pbdt-lacZ*
R89_E	ACTCCTGCCGTTTGAGGCGCGACGTACC	to generate P*bclA*3 mutations in *pbdt-lacZ*
R117_E	AGGTAAGGCCAGGGGCCTTGTCGCGCAA	to generate P*bclA*3 mutations in *pbdt-lacZ*
F131_E	CCCTGGCCTTACCTAATAATCCACAGGAGAAAA	to generate P*bclA*3 mutations in *pbdt-lacZ*
F103_F	CAAACGGCAAACGTTCTAGCGACAAGGC	to generate P*bclA*3 mutations in *pbdt-lacZ*
R117_F	AGGTAAGGCCAGGGGCCTTGTCGCTAGA	to generate P*bclA*3 mutations in *pbdt-lacZ*
F103_G	CAAACGGCAAACGTTTGCGCAGAAAGGC	to generate P*bclA*3 mutations in *pbdt-lacZ*
R117_G	AGGTAAGGCCAGGGGCCTTTCTGCGCAA	to generate P*bclA*3 mutations in *pbdt-lacZ*

Assessment of promoter activity on agar plates

Bacterial overnight cultures were washed and adjusted in 0.9 % NaCl to an OD_{600} of 1.0. Of this cell suspension, 10 µl was spot inoculated on the plate to be tested. Media used were: AB minimal medium (Clark and Maaloe, 1967), supplemented with a carbon source of 10 mM sodium citrate (ABC), 0.4 % mannose (ABM), 0.4 % glucose (ABG) or 0.4 % mannitol (AB-ol); Luria-Bertani (LB, Difco); or ***P**seudomonas* **I**solation **A**gar (PIA, Difco). Media were supplemented with 50 µg/ml 5-Bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal, Gold Biotechnology, USA). Plates were incubated at 37 ºC for two days and then kept at room temperature. Blue colouration of the bacterial colonies was indicative of promoter activity.

Chromosomal *lacZ* insertion

To replace *bclA* with an in-frame *lacZ* insertion, a modified protocol established by Flannagan *et al*. was used (Flannagan *et al.*, 2008). Left (Primers: AS109, AS110) and right (Primers: AS111, AS112) homology arms of *bclA* were amplified with Phusion Polymerase from genomic *B. cenocepacia* H111 DNA. Both amplicons were individually cloned into pGEM®-T Easy vector (Promega). *E. coli* DH5α was transformed with the resulting plasmid, and checked by sequencing with primers: M13_F and M13_R. Purified plasmids were digested with restriction enzymes as follows: for homology arm 1 (109.110); EcoRI and PstI and for homology arm 2 (111.112); XbaI and KpnI. The first ligation was performed with the EcoRI and PstI linearized vector pGPI-SceI-MCS2-*lacZ* and the 109.110 insert. The ligation product pGPI-*lacZ*(109.110) was transformed into *E. coli* SY327λpir, purified, tested by PCR using primers: ES45/ P130, and checked by sequencing. The second ligation was performed with the XbaI and KpnI digested vector pGPI-*lacZ*(109.110) and the 111.112 insert. The ligation product pGPI-*lacZ*(109.110/ 111.112) was transformed into *E. coli* SY327λpir, purified, tested by PCR with primers: ES44/ P129, and introduced into *B. cenocepacia* H111 by conjugation. Transformants were selected on PIA containing 100 μg/ml trimethoprim and were PCR verified. To stimulate a second homologous recombination event, the I-SceI expression plasmid pDAIGm-SceI was introduced into the target strain. Thereby, either the wildtype was restored, or the sequence between the two flanking regions was deleted, resulting in an unmarked mutant. Selection was performed on PIA containing 20 μg/ml gentamicin, following purification on PIA. Generation of *B. cencocepacia* H111 *bclA*::*lacZ* was verified by PCR, using primer pair AS78/ P130.

Generation of *lacZ* promoter fusions

DNA fragments containing the promoter fragments were PCR amplified from *B. cenocepacia* H111 genomic DNA, unless otherwise stated. For transcriptional fusions into pSU11Tp, primers were used for each construct as indicated in parentheses: P*bclA*1-*lacZ* (AS83/ 49), P*bclA*2-*lacZ* (AS50/ 49), P*bclA*3-*lacZ* (AS78/ 49), P*bclA*4-*lacZ* (AS83/81), P*bclA*7-*lacZ* (AS78/ 84). For the translational fusion into pbdt-*lacZ*, primers were used for the construct as indicated in parentheses: P*bclA*3-*lacZ* (AS132/ 133). The PCR fragments were cloned into the pGEM®-T Easy vector (Promega) and in turn checked by sequencing. The plasmid was digested and cloned via the restriction sites XhoI, HindIII of pSU11Tp or EcoRI, NcoI of pbdt-*lacZ*. The generated plasmids were designated as pSU-A1 to pSU-A7 and pbdt-A3,

respectively. Constructs were tested by PCR using the primers AS79 and AS80, and where appropriate, inserts were checked by sequencing. Plasmids were transferred to *B. cenocepacia* by triparental mating.

In order to generate mutated fragments of pbdt-A3, assembly PCR was performed according to the protocol of Rouillard *et al.* with minor modifications (Rouillard *et al.*, 2004). First, an overlapping set of oligonucleotides to represent the promoter region A3 and flanking restriction sites (EcoRI, NcoI) was created using the online Gene2Oligo server (Rouillard *et al.*, 2004). A total of 14 oligonucleotides with sizes ranging from 14 to 22 nucleotides, and an average Tm of 63.92 ºC were software-generated as indicated in Table 3.4. For each mutated fragment the corresponding primers were exchanged for oligonucleotides carrying the desired mutation. Assembly PCR was run for each mutated fragment, followed by a second round of PCR as described in (Rouillard *et al.*, 2004) with primer pair F0 and R179. The second round PCR product was purified and cloned into pbdt-*lacZ*.

Table 3.4 List of oligonucleotides (Microsynth AG, Balgach) used for mutation of the P*bclA*3 promoter region. For each mutated P*bclA*3 fragment (A-G) 13 or 14 oligonucleotides were used for assembly PCR. Oligonucleotide sequences are listed in Table 3.3.

	A	B	C	D8	E	F	G
1	R0	R0	R0	R0	R0	R0	R0
2	F0	F0	F0	F0	F0	F0	F0
3	R15	R15	R15_51	R15_51	R15_51	R15_51	R15_51
4	R51	R51					
5	F33	F33	F33	F33	F33	F33	F33
6	F67	F67	F67	F67	F67	F67	F67
7	R89	R89	R89	R89d2	R89_E	R89	R89
8	R145	R145	R145_C	R145	R145	R145	R145
9	F164	F164	F164	F164	F164	F164	F164
10	R179	R179	R179	R179	R179	R179	R179
11	R193	R193	R193	R193	R193	R193	R193
12	F103	F103	F103_c	F103d2	F103_E	F103_F	F103_G
13	R117	R117b	R117_C	R117d	R117_E	R117_F	R117_G
14	F131	F131b	F131_C	F131	F131_E	F131_E	F131_E

Generation of pAS-1

The 5' leader region of *bclACB* was PCR amplified using genomic DNA from *B. cenocepacia* H111 as template, and primers AS150 and AS151. The *lacZ* promoter region was PCR amplified using the vector pBBR1MCS-5 as template, and primers AS148 and AS149. Both fragments were subcloned into pGEM®-T Easy vector (Promega) and checked by

sequencing. Each fragment was excised using the restriction endonucleases KpnI and NcoI (5' *bclA* leader region) or PstI and KpnI (*lacZ* promoter region). A tripartite ligation was performed using both fragments and the PstI, NcoI linearized vector pbdt-*lacZ*.

Pathogenicity assay using *Caenorhabditis elegans*

C. elegans strains were cultivated on nematode growth medium (NGM), and seeded with *E. coli* OP50 as described. Analysis of toxicity from bacterial strains to *C. elegans* was performed as previously described (Künzler *et al.*, 2010). All *C. elegans* strains were kindly provided by Dr. Markus Künzler, ETHZ. BclB and BclB N-terminal domain were recombinantly expressed in *E. coli* BL21(DE3) as previously described (Inhülsen *et al.*, 2012). Briefly, bacteria were grown in LB medium to an OD_{600} of 1.0 at 37 ºC, with 225 rpm shaking. 1 mM IPTG was added and cultures were grown overnight at 30 ºC. The following day, cells were harvested, washed in phosphate-buffered saline PBS and the OD_{600} was adjusted to 2.0. A culture sample was checked for protein expression by SDS-PAGE (protocol in the Material and Methods section of Chapter 4). Of the bacterial suspension, 80 µl was mixed with 20 µl of L1 worms (10-30), that had been synchronised by egg prep as described elsewhere (Stiernagle, 2006). After 48 h, the developmental stage of the worms was determined.

Measurement of β-galactosidase activities

Cells for analysis of β-galactosidase were inoculated in LB broth containing the appropriate antibiotics, to an OD_{600} of 0.05 from washed overnight cultures. Cells were grown at 37 ºC, 225 rpm to OD_{600} of 2.3-2.8, unless otherwise stated.

β-galactosidase activity was measured as previously described, with some modifications (Inhülsen *et al.*, 2012). Briefly, the cell pellet was resuspended in 500 µl Z-buffer (40 mM NaH_2PO_4 x H_20, 60 mM Na_2HPO_4 x 2 H_2O, 10 mM KCl, 1 mM $MgSO_4$, pH= 7.0, 50 mM β-Mercaptoethanol), 25 µl of 0.05 % SDS and 25 µl $CHCl_3$ was added. The suspension was vortexed for 5 sec and incubated at 30 ºC for 10 min. Then, 200 µl of ONPG (4 mg/ml in Z-Buffer) was added and time was measured until the reaction was stopped with 250 µl 1 M Na_2CO_3, after development of a suitable yellow colour. The sample was centrifuged at 4 ºC, 15 min at 13000 rpm. OD_{420} and OD_{550} were determined, then β-galactosidase activity was calculated as described elsewhere (Aguilar *et al.*, 2014).

Chapter 4

Identification of LepR as a novel regulator of EPS production and lectin biosynthesis in *Burkholderia cenocepacia* H111

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4.1 Introduction

In *Burkholderia cenocepacia* H111 the cell density-dependent regulation of virulence genes and biofilm formation is controlled by two quorum sensing (QS) systems. These QS systems are based on the synthesis, release and detection of two different classes of signal molecules: *N*-acyl-homoserine lactones (AHLs) and *cis*-2 dodecenoic acid (BDSF) (Gotschlich *et al.*, 2001; Deng *et al.*, 2012; Inhülsen *et al.*, 2012). Both AHL- and BDSF-dependent QS systems regulate a specific as well as an overlapping set of genes. Both systems were shown to be involved in the regulation of biofilm formation, protease production, swarming motility and pathogenicity (Schmid *et al.*, 2012; Suppiger *et al.*, 2013). Among the genes regulated by both QS systems is the lectin operon *bclACB*, the large surface protein *bapA* and the gene clusters *bce-I* and *bce-II*, required for production of the exopolysaccharide (EPS) cepacian. It was hypothesized that a BDSF-dependent c-di-GMP effector either controls gene expression directly and in parallel to the AHL-based QS system and/ or it converges with the AHL-based system via a common regulator, which in turn regulates expression of target genes, however the mechanism is still unknown (Schmid *et al.*, 2012).

Bacterial lectins show strong, specific affinity towards their carbohydrate ligands. In *B. cenocepacia* H111, the genes encoding three soluble lectins are organized in an operon, *bclACB* (I35_4184-82). The BclACB lectins have been shown to be involved in virulence by altering biofilm formation and it has been suggested that they are important for cell recognition by binding the LPS of *Burkholderia* species[5] (Inhülsen *et al.*, 2012; Marchetti *et al.*, 2012).

EPS are high-molecular weight sugar-based polymers that are secreted by many microorganisms (Ferreira *et al.*, 2011). Members of the genus *Burkholderia* produce at least seven different types of EPS, with some strains producing only a single type and others producing mixtures (Ferreira *et al.*, 2011). The EPS most commonly produced by *Burkholderia* is cepacian (Hallack *et al.*, 2010; Cuzzi *et al.*, 2014), which has been characterised as a branched heptasaccharide repeating unit composed of 5 sugars; glucose, mannose, galactose, rhamnose and glucuronic acid (Cescutti *et al.*, 2000). Genes involved in the biosynthesis of cepacian are located within two gene clusters *bce-I* and *bce-II* (Moreira *et al.*, 2003; Ferreira *et al.*, 2010). Both clusters are present in all *Burkholderia* genomes sequenced to date, except in *Burkholderia rhizoxynica* HKI 454, in which both gene clusters are absent, and *Burkholderia*

[5] Detailed characteristics for each lectin BclACB are given in Section 1.4.2.

mallei which only harbours *bce-II* (Ferreira *et al.*, 2011). The two bce gene clusters are physically separated in many *Burkholderia* strains including the Bcc species, but are adjacent to each other in a few *Burkholderia* strains (Ferreira *et al.*, 2010).

In this study, we identified a novel regulator, I35_4766, designated as LepR (**le**ctin and **EP**S regulator) that is required for expression of the lectin operon *bclACB* and production of the EPS cepacian. We provide evidence that LepR is involved in the regulation of various phenotypes, including protease production, colony morphology and pellicle formation. By employing RNA-Seq we found that LepR is a global regulator that controls expression of genes known to be regulated by the two QS systems operating in this strain or by RpoN. Our results suggest that LepR is a novel transcriptional regulator, that is partially integrated in the QS system and controls expression of potential functionally linked genes in *B. cenocepacia* H111.

4.2 Results

4.2.1 Identification of genes affecting expression of the *bclACB* lectins

In order to identify genes affecting the BDSF signal transduction cascade, the *cis*-2 fatty acid specific biosensor pSU11 P_{bclA}-*lacZ* (Inhülsen *et al.*, 2012; Suppiger *et al.*, 2016a) was conjugated into a *B. cenocepacia* H111 Tn*5* transposon insertion library (Christian Jenul, laborarory collection). Of approximately 20,000 transposon mutants screened, 24 showed lowered and 5 showed increased P_{bclA}-*lacZ* activity (SI 4.4.1, Table S 4.1). The insertion sites in these mutants were determined by sequencing the regions flanking the transposon. In three of the mutants, which showed reduced β-galactosidase activity, the transposon had interrupted the BDSF receptor gene *rpfR* (Schmid *et al.*, 2012), confirming the suitability of the genetic screen (Table S 4.1).

Three mutants with reduced *bclACB* promoter activity were found to carry the transposon within I35_4766 (homologous to J2315BCAM0853), designated as *lepR* (**l**ectin and **EP**S regulator). Interestingly, the same gene was also identified in an independent screen for mutants that formed smooth colonies when BCAM1349, the master regulator of the *bmp* exopolysaccharide biosynthesis cluster, was overexpressed (Tim Tolker-Nielsen, personal communication). Given that expression of the Bmp polysaccharide is also BDSF-regulated (Elisabeth Steiner *et al.*, in preparation), we focussed our efforts on the characterization of

this putative regulator, which may link BDSF signalling with exopolysaccharide expression and lectin production.

The *lepR* gene product consists of 313 amino acids, with a predicted molecular weight of 35.2 kDa and is annotated as a hypothetical protein. NCBI domain analysis (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) identified conserved domains with homology to DUF4096 (pfam13340, putative transposase of IS4/5 family, E-value 6.52e-19; amino acids 6-81) and of HlyU (pfam10115, transcriptional activator, E-value 8.86e-04; amino acids 186-256) with a very low probability. In addition, the protein structure and function of LepR was analysed using the phyre2 server (Kelley *et al.*, 2015). The prediction, based on alignment with the DNA-binding domain of *E. coli* Lrp, suggested the presence of a weak DNA-binding site within LepR. The predicted 3D structure of LepR is shown in Figure 4.1. An inverted repeat (CCGGCGCGTCG) was identified within *lepR* at position 657-667 (+strand) and 768-778 (-strand).

Blast search revealed that LepR homologs are present in most *Burkholderia* species, including the Bcc, the *B. pseudomallei* group and the plant-beneficial environmental strains (Table 4.1). All genes identified that had similarity to LepR were located next to *bceA* or homologs. BceA is a bifunctional protein with phosphomannose isomerase and GDP-mannose pyrophosphorylase activities and is part of the cepacian cluster *bce-I* (Sousa *et al.*, 2007a). No LepR homolog was identified in *Burkholderia rhizoxynica* HKI 454, which lacks both *bce-I* and *bce-II*. In *Burkholderia mallei*, a species which is lacking *bce-I* (Moreira *et al.*, 2003), a LepR homologue was only found where a pseudogene with similarity to *bceA* was present (present in 16 out of 30 *B. mallei* strains published at NCBI). No LepR homologs could be identified in bacteria not belonging to the genus *Burkholderia*.

Figure 4.1 Predicted 3D structure of LepR. Protein structure was predicted by the PHYRE 2 server (Kelley *et al.*, 2015). Red indicates high confidence, blue low.

Table 4.1 List of LepR homologs in *Burkholderia*.

Group[a]		Strain	% query cover[b]	% identity LepR[b]	LepR homolog Locus Tag[c]	LepR homolog Ref seq	next to *bceA* homolog[d]
Pathogenic group	Bcc	*B. ambifaria* AMMD	100	85	BAMB_RS27640	WP_011660454.1	+
		B. cenocepacia J2315	100	100	Bcam0853	WP_006482942.1	+
		B. cenocepacia MCO-3	100	96	BCENMC03_RS18745	WP_011548200.1	+
		B. cenocepacia HI2424	100	96	Bcen2424_3818	WP_011548200.1	+
		B. cepacia DDS 7H-2	100	100	DM42_RS08625	WP_006482942.1	+
		B. cepacia GG4	100	86	GEM_RS24385	WP_014900076.1	+
		B. contaminans	100	89	n.a.	WP_039345296.1	n.a.
		B. dolosa PC543	100	84	BDSB_RS15595	WP_035973608.1	+
		B. lata 383	100	90	BCEP18194_RS34120	WP_011355871.1	+
		B. multivorans ATCC	100	83	BMUL_RS24825	WP_012217475.1	+
		B. pyrrocinia Lyc2	100	91	JM78_RS34515	WP_034185151.1	+
		B. ubonensis Bu	100	78	BUBO0001_RS00830	WP_010089089.1	+
		B. vietnamiensis G4	100	84	Bcep1808_4199	WP_011881531.1	+
	Pseudomallei group	*B. pseudomallei* ABCPW 111X946	100	69	X946_RS12685	WP_038748019.1	+
		B. pseudomallei BPC006	100	68	BPC006_RS27995	WP_004529088.1	+
		B. thailandensis MSMB121	100	68	BTI_RS25765	WP_015603844.1	+
		B. mallei ATCC 10399	100	67	BMA10399_RS16565	WP_004190269.1	pseudo-gene bceA
		B. oklahomensis EO147	100	67	DM82_RS30800	WP_010110227.1	+
	PP	*B. glumae* PG1	100	49	BGL_RS28920	WP_042628562.1	+
		B. glumae BGR1	100	50	BGLU_RS25820	WP_015877678.1	+
		B. sordidicola S170	79	46	FH08_RS0134885	WP_031362772.1	(+)
		B. gladioli BSR3	100	48	BGLA_RS30530	WP_013690949.1	+
non-pathogenic group	PBE	*B. sprentiae* WSM5005	83	50	BUR5005_RS0125030	WP_027197720.1	+
		B. acidipaludis NBRC101816	96	50	BAC01S_RS32665	WP_043203808.1	+
		B. mimosarum NBRC106338	84	47	BMI01S_RS21595	WP_036044481.1	(+)
		B. dilwothii WSM3556	80	45	F759_RS0104020	WP_027798720.1	+
		B. nodosa DSM21604	84	45	A3CW_RS45610	WP_035512585.1	(+)
		B. fungorum NBRC102489	84	48	BFU01S_RS16170	WP_028196547.1	+
		B. ginsengisoli NBRC100965	84	47	BGI01S_RS06985	WP_042323613.1	+
		B. kururiensis M130 Bk_3	84	48	G118_RS32975	WP_033350699.1	+
		B. phenoliruptrix AC1100	81	43	BPAC_RS23830	WP_035482608.1	+
		B. phymatum STM815	69	65	BPHY_RS05355	YP_001857290.1	+
		B. phytofirmans PsJN	83	50	BPHYT_RS09700	WP_012432961.1	+
		B. xenovorans LB400	80	49	DR64_RS21655	WP_011488340.1	+

a. Group: Bcc, *Burkholderia cepacia* complex; PP, plant pathogens; PBE, plant beneficial environmental strains.

b. LepR homologue identity according to online protein blast search (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi by nr- non redundant search), n.a. not applicable.

c. Locus tag according to the *Burkholderia* database (http://burkholderia.com/blast/set), n.a. not applicable.

d. +, *lepR* chromosomally located next to a *bceA* homolog (e.g. *cpsB*), identified by the *Burkholderia* database; (+), at present not annotated, but blast search revealed identity to *bceA*.

4.2.2 Construction and characterization of a defined LepR mutant

For further analysis, an unmarked deletion mutant of *lepR* was constructed by employing a mutagenesis system that is based on the homing endonuclease I-SceI, using the protocol of Flannagan *et al.* (Flannagan *et al.*, 2008). Briefly, two regions flanking *lepR* were PCR-amplified from genomic *B. cenocepacia* H111 DNA. These amplicons were cloned into the suicide vector pGPI-SceI and the resulting plasmid, was introduced into *B. cenocepacia* H111 and *B. cenocepacia* H111-rpfF by conjugation. Following this, the I-SceI expression plasmid pDAIGm-SceI was introduced and *B. cenocepacia* H111-lepR was distinguished from *B. cenocepacia* H111 by PCR profiling.

For genetic complementation, the gene *lepR* was PCR amplified from genomic *B. cenocepacia* H111 DNA and cloned into pBBR1MCS-5, generating pBBR(lepR). The vector pBBR(lepR) was transferred to *B. cenocepacia* strains by triparental mating.

To confirm the involvement of LepR in the regulation of the *bclACB* operon, we measured transcriptional activities of a P*bclA-lacZ* fusion (Inhülsen *et al.*, 2012) in the wild-type and *lepR* mutant backgrounds. β-galactosidase activity was found to be approximately 2.3-fold reduced in the mutant relative to wildtype (Figure 4.2A). Furthermore, western blot analysis (Figure 4.2B) of cell extracts from the wildtype, *lepR* mutant and complemented mutant H111-lepR/pBBR(lepR), using anti-BclB antibodies, revealed that expression of BclB had diminished in H111-lepR, but not in the complemented strain H111-lepR/pBBR(lepR).

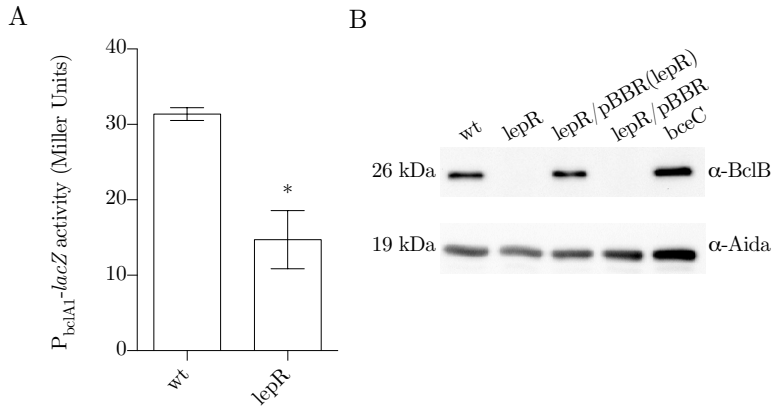

Figure 4.2 LepR controls expression of the lectin operon *bclACB*. A, *bclA* promoter activity of pSU11 PbclA-*lacZ* was measured in both the wildtype and the *lepR* mutant backgrounds. Error bars represent SD. Asterisks denote significant difference in *lacZ* expression (t-test, * $p<0.05$, n=2). **B**, Expression of *bclB*, but not *aidA* was reduced in H111-lepR. Western Blot analysis using antibodies against BclB and AidA was performed on cell extracts grown for 24 h on NB plates. As a vector control, the *lepR* mutant complemented with the empty vector pBBR1MCS-5 and as a positive control, the mutant H111-bceC (which was disrupted in gene I35_4769 of bce-I, Bcam0855 in J2315) was included.

4.2.3 Expression of LepR is regulated by RpoN and RpfF

To investigate expression of LepR, an in-frame translational fusion of this gene to *lacZ* was constructed. This construct, named pbdt-*lacZ*, was introduced into the wildtype *B. cenocepacia* H111 and various mutant strains. Expression of this fusion was significantly down-regulated in the H111-rpoN and H111-rpfF mutant backgrounds (Figure 4.3). This is consistent with a previous transcriptomic analysis, that showed that expression of *lepR* is RpoN-dependent (Lardi *et al.*, 2015). *In silico* analysis of the *lepR* promoter region identified a putative RpoN binding site (TGGTATGCTTCGTGCA) 211 bp upstream of the translation start site (TSS). Regulation of *lepR* by RpfF has also been reported in another RNA-Seq analysis (Schmid *et al.*, 2012). Expression of *lepR* was not significantly altered in the *cepI* and *cepR* mutants, suggesting that *lepR* is not regulated by the AHL-based QS system.

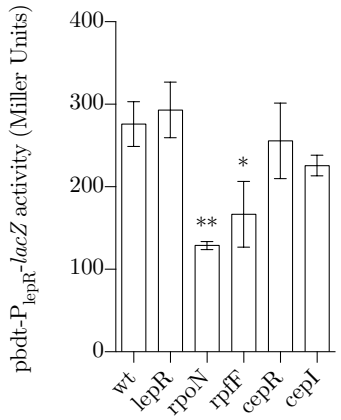

Figure 4.3 Expression of *lepR* is RpoN- and RpfF-regulated. The promoter region of *lepR* was cloned in front of a promoter-less *lacZ* reporter gene in pbdt-lacZ and in turn introduced into different genetic backgrounds of *B. cenocepacia* H111. β-galactosidase activities were measured using culture samples taken in the late exponential growth phase at an OD_{600} = 2.0-2.6 (ANOVA, * $p < 0.05$; Bars, SD, $n \geq 2$).

4.2.4 LepR is required for EPS production and influences the macrocolony morphotype

The results of the transposon screen (Section 4.2.1 and 4.4.1) provided strong evidence for a regulatory role of LepR in the expression of the lectin operon. Previously, lectin production had been shown to be controlled by the BDSF- and AHL-dependent QS systems (Inhülsen *et al.*, 2012; Schmid *et al.*, 2012). In order to identify possible overlaps between these systems, the *lepR* mutant was tested for various well characterized QS-regulated phenotypes.

The results of a phenotypic characterization of the in-frame deletion mutant H111-lepR and the complemented mutant H111-lepR/pBBR(lepR) are depicted in Figure 4.4. EPS production on YEM agar plates was completely abolished in the *lepR* mutant, but was restored in the complemented mutant. In *B. cenocepacia* H111, both cepacian clusters (I35_4767-77 (*bce-I*) and I35_4922-29 (*bce-II*) are required for maximal production of cepacian, suggesting that both clusters are less expressed in H111-lepR than in the wildtype (Figure S 4.1). While the *B. cenocepacia* H111 wild-type strain forms a thin and fragile pellicle at the liquid-air interface in NYG broth, the LepR mutant formed a thick pellicle under static conditions, similar to a pellicle formed by an *rpfR* mutant (data not shown). Upon genetic complementation with pBBR(lepR), the pellicle strength was reduced to the level of the wild-type strain. The structure of macrocolony biofilms was analysed on NYG agar plates. While the mutant LepR formed flat colonies, the complemented strain grew similarly to the wild-type strain, but was slightly stronger in the centre of the colony (Figure 4.4 and Figure S 4.2). This latter effect might have been observed because expression of *lepR* in pBBR(lepR) is under the control of both its native, and the *lac* promoter, and may therefore exceed biological levels. These results not only indicate the involvement of LepR in colony morphogenesis, but also that its activity may depend on specific growth conditions (i.e. location within a macrocolony). Further information on the involvement of LepR in wrinkly colony formation is given in Figure S 4.2. The ability of the mutant to form static biofilms in microtiter plates was unchanged relative to the wildtype (Figure 4.5).

Extracellular protease production was unaffected in the *lepR* mutant (Figure 4.4). However, the complemented mutant produced decreased amounts of extracellular protease. This effect was also observed when pBBR(lepR) was introduced into the *B. cenocepacia* H111 wild-type strain (Figure S 4.3). Swarming motility of the LepR mutant was indistinguishable from that of the wild-type strain. Interestingly, however, when pBBR(lepR) was introduced into the non-swarming *rpfR* mutant, swarming motility was rescued (data not shown). Although the underlying molecular mechanism remains to be elucidated, these results

suggest that LepR affects swarming motility by interacting with a component of the RpfR signalling cascade.

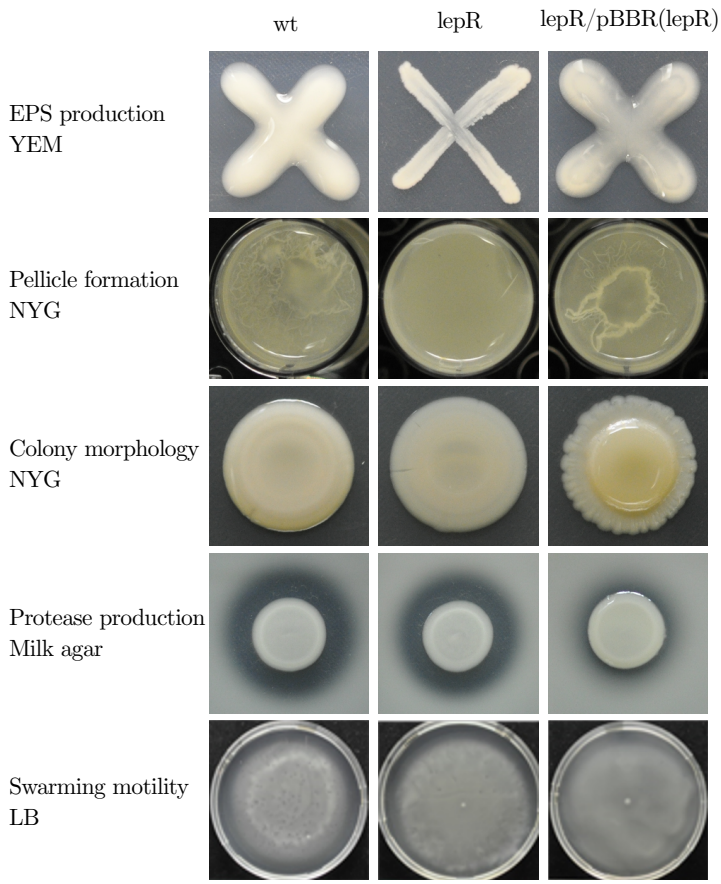

Figure 4.4 LepR is involved in the regulation of EPS and protease production, pellicle formation and colony morphogenesis. The *B. cenocepacia* H111 wild type (wt), the *lepR* mutant (H111-lepR) and the complemented mutant (H111-lepR/pBBR(lepR)) were analysed for the expression of various phenotypes as described in the Materials and Methods. Data shown are representative pictures from at least three replicates.

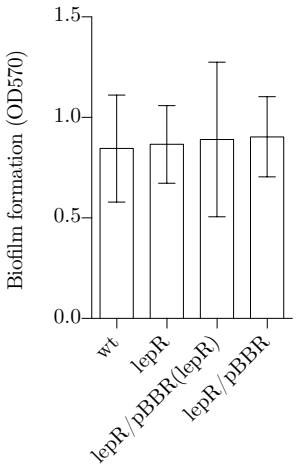

Figure 4.5 LepR is not involved in biofilm formation. Biofilm formation in AB-citrate medium under static conditions was measured after 48 h of growth in 96-well plates. Shown are mean values with SD, n=3.

4.2.5 LepR is a novel global regulator

To investigate the role of LepR in global gene regulation in *B. cenocepacia* H111, we performed RNA-Seq on the *lepR* mutant, the complemented mutant and the H111 wildtype. RNA was extracted from cultures harvested at an OD_{600} of 2.0-2.2. In total, 73 genes were found to be differentially expressed in the mutant and 201 genes in the complemented mutant, relative to the wild-type strain (FC≥3, p<0.12; Table S 4.2).

The RNA-Seq analysis confirmed the regulation of the *bclACB* operon by LepR. Additionally, expression of both cepacian clusters, I35_4767 to I35_4778 (*bce-I*, except *bceG*) and I35_4922 to I35_4930 (*bce-II*), were down-regulated in the mutant strain, which is in line with the observed reduction in EPS production. Analysis of the RNA-Seq profile of the complemented mutant showed that LepR is also involved in the regulation of transcription of the ZmpA protease (3-fold down-regulated in the complemented strain) and genes involved in motility (4 genes, including *fliC*, *flgL*, *motB*), consistent with the observed phenotypes. Interestingly, analysis of the RNA-Seq data revealed negative regulation of *rsaM* by LepR (3.42-fold up-regulated in the mutant). *rsaM* is located in the intergenic region between *cepR* and *cepI* and was previously suggested to encode a downstream regulator of the CepIR-dependent QS system (Inhülsen, 2011).

Other genes regulated by LepR are involved in amino acid- (19 genes) or carbohydrate- (9 genes) transport and metabolism. The genes I35_0827-8 and I35_0830 encode part of a monosaccharide ABC transporter for ribose/ autoinducer 2/ D-xylose or glucose/mannose. This down-regulation of carbohydrate transporters in the complemented mutant is consistent with observed growth deficiency of H111-lepR/pBBR(lepR) and H111/pBBR(lepR) in minimal medium (AB) supplemented with D-mannose, L-rhamnose or D-xylose (data not shown). It is not clear whether this down-regulation of sugar transporters affects cepacian production. Further analysis of the LepR regulon identified genes involved in translation, ribosomal structure and biogenesis (19 genes) and transcription (6 genes). Expression of genes encoding ribosomal proteins was found to be up-regulated in the complemented mutant relative to the wildtype. Furthermore, the complemented mutant showed increased expression of genes encoding subunits of the F-type ATPase and proteins of the oxidative phosphorylation and proton-motive force-driven ATP biosynthesis (*atp*, I35_0032-36).

Of 150 RpfF-regulated genes, 34 (22.6 %) were also found to be regulated by LepR. Among these are the *bce-I* and *bce-II* clusters, the lectin operon *bclACB* and the flagellin gene *fliC*. 12 out of 158 (7.59 %) CepR-regulated genes were also regulated by LepR. Interestingly, we also observed an overlap with the RpoN regulon: 44 of the 276 (15.9 %) RpoN-regulated genes (Lardi *et al.*, 2015) were found to be differentially expressed in the *lepR* mutant.

4.2.6 LepR is involved in persister cell formation

Alteration of oxidative phosphorylation and metabolism was previously shown to be critical for bacterial persistence (Lewis, 2005; Van Acker *et al.*, 2013; Marques *et al.*, 2014). As we observed increased expression of *atp* genes and genes encoding ribosomal proteins in the complemented mutant, we tested whether expression of LepR influences the abundance of persister cells. We performed the analysis by exposing late stationary phase *B. cenocepacia* H111 cells to ciprofloxacin, an antibiotic of the fluoroquinolone class that inhibits DNA gyrase. Exposure to ciprofloxacin or ampicillin is a standard procedure for persister cell isolation (Marques *et al.*, 2014). A mutation in *lepR* increased the number of persisters after treatment with ciprofloxacin, while overexpression of *lepR* (H111 wt/pBBR(lepR) or H111-lepR/pBBR(lepR)) decreased the number of persisters compared to the empty vector control (Figure 4.6). We also observed an increased number of persister H111-lepR cells relative to the wild-type strain.

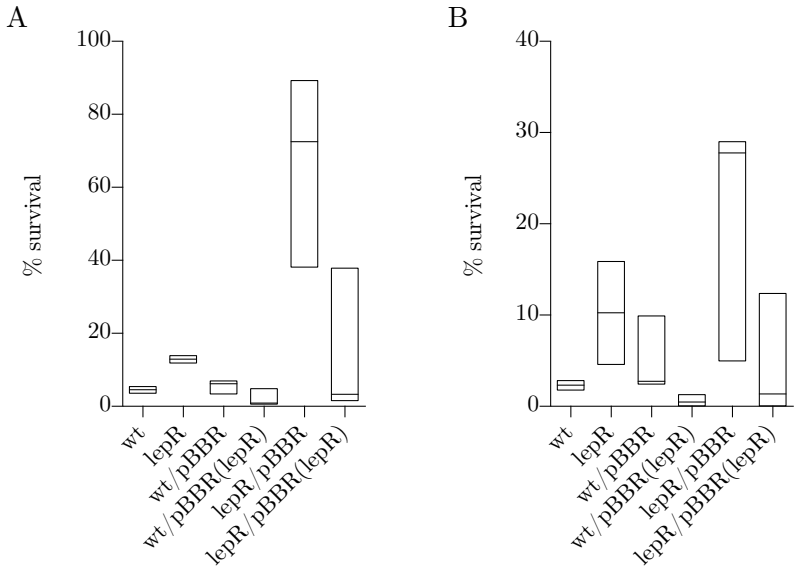

Figure 4.6 Expression of *lepR* influences the number of persister cells. Stationary phase planktonic cultures were exposed to 10 (**A**) or 50 (**B**) µg/ml ciprofloxacin in saline for 20 h. CFUs were determined after recovery on LB plates for 24 h. Line in bars (min to max) represent median n ≥2.

4.2.7 LepR neither influences the intracellular c-di-GMP level nor AHL or BDSF production

In order to test whether LepR affects the production of signal molecules, we measured the concentrations of c-di-GMP, AHL and BDSF. Intracellular c-di-GMP levels of the *B. cenocepacia* wild-type H111, the *lepR* mutant and the complemented mutant were indistinguishable (Figure 4.7A). AHL and BDSF production was analysed with the aid of the *cis*-2 unsaturated fatty acid biosensor (*B. cenocepacia* H111-rpfF/pAN-L15) and the C8-AHL biosensor *P. putida* F117/pAS-C8, respectively. Neither the BDSF nor the AHL level was altered in the mutant or the complemented strain (Figure 4.7B, C). These results are in agreement with our RNA-Seq data, where neither *cepI* nor *rpfF* was found to be regulated by LepR.

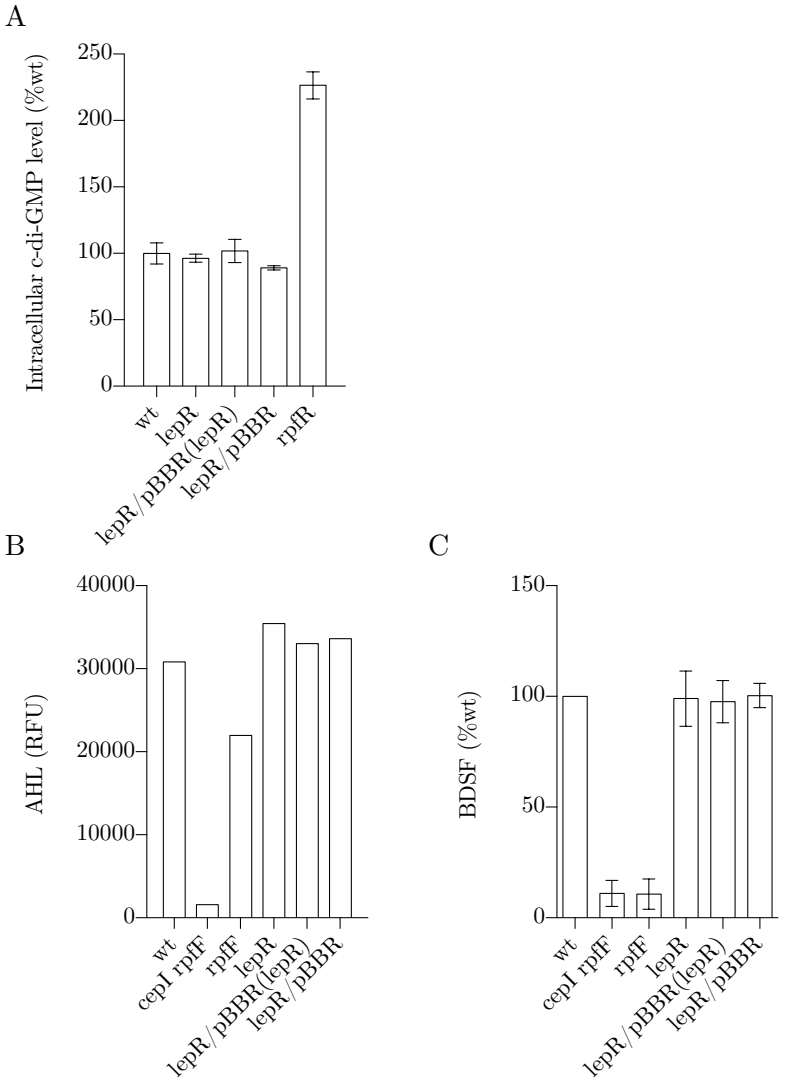

Figure 4.7 LepR neither affects intracellular c-di-GMP level nor AHL- or BDSF biosynthesis in *B. cenocepacia* H111. A, c-di-GMP was extracted from cultures grown to late exponential growth phase in LB. Intracellular c-di-GMP levels in H111-derived strains bearing null mutations in the gene/genes shown were compared to *B. cenocepacia* H111 and normalized to OD_{600}. Shown are mean values with SD, n=2 (2 biological and 2 technical replicates). **B**, AHL- and **C**, BDSF production was measured with the aid of biosensors. Shown are mean values with SD, n=3.

4.2.8 LepR is not a downstream regulator of RpfR

We next performed experiments to investigate whether the overlap of the RpfF and LepR regulon is a consequence of LepR being a downstream regulator of the BDSF-dependent signalling cascade. To this end, the plasmid pAS-1 was introduced into an *rpfF lepR* double mutant as well as into the single mutants and the wild-type strain. On plasmid pAS-1, the leader sequence (5'untranslated region, UTR) of the *bclACB* lectin operon is fused upstream of the *lacZ* reporter gene and downstream of the *lac* promoter (Chapter 3). Previous experiments had shown that this 5'UTR is sufficient for BDSF-induced and RpfR-dependent expression of the lectin operon (Section 3.2.4). Reduced *lacZ* expression was observed in the H111-rpfF and H111-rpfF lepR mutant backgrounds but not in H111-lepR compared to the wildtype (Figure 4.8A). Upon exogenous addition of BDSF to the medium, β-galactosidase activity was restored in the H111-rpfF and H111-rpfF lepR mutant backgrounds, indicating that RpfF but not LepR influences *bclACB* expression via its 5'UTR region. Hence, LepR is not a downstream regulator of the BDSF signalling pathway.

In contrast to pAS-1, plasmid pbdt-A3 contains not only the 5'UTR region but also the -35 and -10 region of the native *bclACB* promoter in front of *lacZ*. In H111-rpfF lepR and H111-rpfF carrying the plasmid pbdt-A3 no significant *lacZ* activity was observed. However, upon addition of BDSF to the medium, *lacZ* expression was restored in H111-rpfF, but not in the double mutant H111-rpfF lepR (Figure 4.8B). These results show that LepR influences gene expression by modulating the *bclACB* promoter activity.

Taken together, these results indicate that i) LepR is not part of the BDSF signalling pathway, but ii) LepR is required for expression of the lectin operon by (indirectly) interacting with a 66 bp region, overlapping the -35 and -10 promoter of *bclACB*.

In order to confirm these results, conditional mutants of *lepR* in the *B. cenocepacia* H111 wild-type strain and the *rpfF* mutant H111-rpfF were constructed, designated *B. cenocepacia* H111 Prha-lepR and H111-rpfF Prha-lepR, respectively. In these strains expression of *lepR* is induced upon addition of L-rhamnose to the medium. Both conditional mutants *B. cenocepacia* H111 Prha-lepR and H111-rpfF Prha-lepR were transformed with pAS-1 and pbdt-A3. β-galactosidase activity of pAS-1 was independent of LepR and was maximal in the presence of BDSF (Figure S 4.4). In the case of plasmid pbdt-A3 both BDSF and LepR were required for full activity.

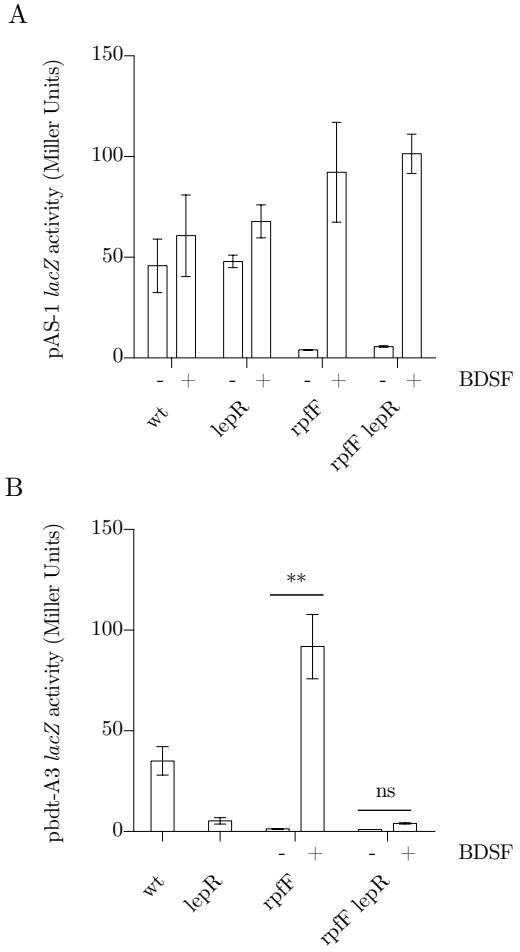

Figure 4.8 LepR controls expression of the lectin operon *bclACB* through a 66 bp region overlapping the -35 and -10 sites of the promoter. A, promoter activity of pAS-1 containing a synthetic *lac* promoter is not affected in the *lepR* mutant background. **B**, Promoter activity of pbdt-A3 carrying the native -35 and -10 promoter of *bclA* is negligible in both single mutants H111-lepR, H111-rpfF and the double mutant H111-rpfF lepR, and in the latter was not activated upon addition of BDSF (10 μM) to the medium. Shown are mean values with SD, n≥2.

4.3 Discussion

Recently, it has been shown that the BDSF- and the AHL-dependent regulons share 31 overlapping genes, which include the lectin operon *bclACB* (Schmid *et al.*, 2012). In order to identify further components involved in the regulation of the lectin operon *bclACB* and to investigate the BDSF-based signalling pathway, we performed a transposon screen. Among the 20 genes identified that showed altered *bclACB* expression was the gene I35_4766, designated as *lepR*, in addition to the BDSF receptor RpfR. In this study we show that LepR is required for both lectin expression and the production of the EPS cepacian. Furthermore, we provide evidence that LepR is a global regulator that is involved in the control of various functions, many of which are related to virulence.

In *B. cencocepacia* H111, *lepR* is located upstream of *bceA*, which is part of the EPS cluster *bce-I* (Sousa *et al.*, 2007a). This chromosomal gene organization is conserved among all sequenced *Burkholderia* genomes, for strains that lack the *bce-I* cluster no LepR homolog was identified. No homologs of LepR were identified in bacteria not belonging to the genus *Burkholderia*.

Regulation of cepacian biosynthesis. In *Pseudomonas aeruginosa* the production of the polysaccharide alginate is regulated by AlgT, RpoN, MucAB and additional transcription factors (Ramsey and Wozniak, 2005). In contrast, in *B. cepacia* RpoE (the homolog of AlgT) doesn't play a role in EPS production (Devescovi and Venturi, 2006), suggesting a different mechanism regulating cepacian production in *Burkholderia*. However, Lardi *et al.* (2015) have shown that RpoN is in fact involved in the regulation of cepacian production in *B. cenocepacia* H111 (Lardi *et al.*, 2015). It also has been shown that mutation of the RNA chaperone Hfq reduces cepacian production in *B. cepacia* IST408 (Sousa *et al.*, 2010) and that the amount and molecular weight of cepacian is post-translationally regulated by bacterial tyrosine kinases (BY-kinases, e.g. BceF) and protein tyrosine phosphatases (PTPs, e.g. BceD) (Ferreira *et al.*, 2007). Furthermore, an AHL-dependent QS system is involved in the regulation of cepacian production in *Burkholderia kuruiensis, Burkholderia xenovorans* and *Burkholderia uname* (Suárez-Moreno *et al.*, 2010) and it has been shown that cepacian production is growth condition dependent (Pellizzoni *et al.*, 2016)[6]. However, the genes directly involved in the regulation of EPS production have not been identified so far. In this study, we show that LepR is required for cepacian production in *B. cenocepacia* H111.

[6] Studies on the transcriptional and post-translational mechanisms affecting cepacian production have been reviewed by Ferreira *et al.* 2011.

Expression of LepR is controlled by RpfF and RpoN, which may ensure that cepacian production is only induced under particular conditions.

The role of cepacian in acute virulence. EPS production was shown to be relevant to virulence, due to its capacity to scavenge reactive oxygen species (Bylund *et al.*, 2006) and to reduce bacteria-macrophage and bacteria-neutrophil interactions (Conway *et al.*, 2004). Several studies show that production of the EPS cepacian is commonly increased concomitantly with the expression of other virulence factors, suggesting a role in pathogenesis (Sousa *et al.*, 2010; Lardi *et al.*, 2015). In support of this hypothesis, Sousa *et al.* have shown that a cepacian mutant of *B. cepacia* IST408 is avirulent in a murine infection model (Sousa *et al.*, 2007b). A recent study analysed the transcriptome of *B. cenocepacia* isolates recovered at the terminal stage of infection of CF patients (Kalferstova *et al.*, 2015). It was found that in bloodstream isolates expression of the T3SS, cepacian and the lectin *bclB* was up-regulated, compared to the expression profiles of sputum isolates before the onset of the cepacia syndrome. These data are in line with our findings that in *B. cenocepacia* H111, expression of the lectin operon *bclACB* and the *bce-I* and *bce-II* gene clusters are co-regulated (by LepR).

Our RNA-Seq data provide evidence that LepR not only controls EPS production and lectin gene expression, but also the expression of genes involved in oxidative phosphorylation and genes coding for ribosomal proteins. Moreover, we have shown that over-expression of *lepR* inhibits the number of persister cells after treatment of cultures with ciprofloxacin. Persister cells are commonly described as dormant, metabolically inactive and tolerant of a wide range of antimicrobials (Keren *et al.*, 2004). Considering that expression of *lepR* is BDSF-dependent regulated, the results by Marques *et al.* are interesting; it has been shown that *cis*-2-decenoic acid increases respiratory activity in biofilm-derived persister cells of *P. aeruginosa* and stimulates expression of proteins involved in translation, protein modification, ATP synthesis and hydrolysis (Marques *et al.*, 2014). For this reason, the fatty acid signalling molecule *cis*-2-decenoic was suggested to be involved in the reversion of persister cells to metabolically active, but antimicrobial-susceptible cells. However, a study of clinical *Burkholderia* isolates showed that both persistence and virulence of respiratory infections depend only partially on EPS production (Cunha *et al.*, 2004).

Additional work will be required to investigate the contribution of LepR to persister formation and virulence in *B. cenocepacia* H111. For example, strains overexpressing LepR have a growth defect in minimal medium, which may affect survival and antibiotic resistance, thereby altering persistence indirectly. Furthermore, cepacian acetylation is an

important protective mechanism which has been suggested to affect persistence (Whitfield *et al.*, 2015). Thus, it must also be considered that LepR may shift the relative expression levels of the two cepacian clusters, such that the level of cepacian acetylation is altered.

In conclusion, our results show that LepR is involved in the regulation of genes that are potentially functionally interrelated: Both lectin *bclACB* expression and cepacian production are supposed to be required for acute virulence, which in turn requires metabolically active cells (resilienced persisters). However, LepR is not part of the RpfR-dependent signalling pathway and appears to control expression of the lectin operon *bclACB* through (indirect) interaction with a 66 bp DNA promoter region. Investigations on the mode of action of the novel regulator LepR and the identification of novel BDSF- and AHL effectors downstream of RpfR and CepR are currently under investigation.

4.4 Supporting Information

4.4.1 Identification of additional genes involved in the regulation of the lectin operon *bclACB*

A screen of approximately 20,000 transposon mutants revealed 29 candidates with lowered, increased or absent P_{bclA}-*lacZ* activity (Table S 4.1). Phenotypic analysis of these candidates was carried out to identify mutants with a phenotype similar to the *rpfF* mutant. For this purpose, biofilm formation experiments, protease activity measurements and lectin expression studies (western blot and β-galactosidase assays) were performed (Table S 4.1). The analysis suggested that genes I35_2688, I35_3197, I35_1915 and I35_0741, I35_0739 and I35_0737 were involved in the regulation of *bclACB,* and are discussed in detail below.

The transposon mutant H111 Tn*5*-2688 was disrupted within gene I35_2688 (bcal2831 in J2315, annotated as a two-component system response regulator *qseB*). The mutant showed increased lectin expression in both β-galactosidase and western blot experiments. Its protease and EPS production was slightly reduced, but biofilm formation, BDSF and AHL production were comparable to wild-type levels. An insertion mutant disrupted in I35_2688 was constructed by introducing pSHAFT2 (internal fragment amplified with the primers AS93/94) and lectin promoter activity was studied by introducing the reporter plasmid pbdt-A3 (Chapter 3). Thereby, increased activity of the lectin promoter in the I35_2688 mutant was confirmed. However, at 42 °C the H111 Tn*5*-2688 mutant was not able to grow in LB medium, in contrast to the wild-type strain. Gene I35_2688 is located on

chromosome 1, next to the gene encoding HtrA protease required for growth under thermal and osmotic stress (Flannagan *et al.*, 2007). Further analysis is required to reveal how I35_2688 is involved in the growth of *B. cenocepacia* H111 at high temperature, its contribution to the regulation of lectin expression and to exclude polar effects. Interestingly, previous experiments have revealed that lectin expression is increased under heat stress (Microarray experiments in *B. cenocepacia* J2315 (Sass *et al.*, 2013)).

Gene I35_3197 (bcal0468 in J2315) encodes the peptidase M48 and I35_1915 (bcae1896 formerly, bcal1994 in J2315) encodes the Lon ATP-dependent protease, previously described to be involved in AHL-dependent QS regulation in *P. putida* and *P. aeruginosa* (Bertani *et al.*, 2007; Takaya *et al.*, 2008). Additionally, the Lon protease was also found to be involved in the virulence of several bacterial species, including *B. cenocepacia* (Schwager *et al.*, 2012).

Gene I35_0737 (encoding a polysaccharide acetylase), I35_0739 (encoding a glycosyltransferase) and I35_0741 (encoding a hypothetical protein) are present (as a putative operon) in the genome of *B. cenocepacia* H111 but not in J2315 (as determined by blastp search on burkholderia.com). In the transposon mutants disrupted within I35_0739 and I35_0737, biofilm formation was increased. However, neither of these genes was found to be regulated by BapR, the major biofilm regulator of *B. cenocepacia* H111 (Aguilar *et al.*, 2014). Protease and EPS production in the mutant disrupted within I35_0739 was not significantly altered. However, in the I35_0737 mutant, both protease and EPS production was reduced. All three genes were found independently to be interrupted in the transposon screen and to alter expression of the lectin operon *bclACB*. Further investigations are required to better understand the contribution of I35_0741, I35_0739 and I35_0737 to gene expression.

Table S 4.1 Genes identified by transposon mutagenesis in *B. cenocepacia* H111 displaying altered promoter activity of the *bclACB* operon, quantified using *lacZ* expression.

	Clone[a]	Colour[a]	Locus ID[b]	Orthologe in J2315[c]	Description	MU_ex %wt[d]	MU_stat %wt[d]	WB[e]	Protease[e]	Biofilm[e]	BDSF synthesis[e]	AHL synthesis[e]	EPS[e]
1	B9	blue	I35_0739	-	Glycosyltransferase	214.9	121.5	up	n.s.	up		yes	(down)
2	E14	dark blue	I35_1915	BCAL1994	ATP-dependent protease La TypeI, *lon*	427.9	464.2	(down)		up	yes	yes	(down)
3	G14	blue	I35_2510/11	BCAL2649	hypothetical Prot/ Segregation and condensation protein A	91.6	69.8						
4	F15	dark blue	I35_2688	BCAL2831	Two-component system response regulator QseB	96.7	260.7	up up	(down)	n.s.	yes	yes	(down)
5	H5	dark blue	I35_3197	BCAL0468	Peptidase M48, Ste24p precursor	2694.6	615.7	up	n.s.	up	yes	yes	n.s.
6	F5	light blue	I35_0737	-	Polysaccharide deacetylase	373.8	70.2	down	down	up	(yes)	yes	(down)
7	C4	light blue	I35_0741	-	hypothetical prot	176.1	86.1	n.s.					
8	E7	light blue	I35_1788	BCAL1870	Transcription accessory protein (S1 RNA-bindingdomain), *tex*			up		n.s.		yes	(down)
9	K10	light blue	I35_1881	BCAL1961	FOG: Ankyrin repeat protein	149.7	64.0	down		n.s.		yes	(down)
10	B12	light blue	I35_2615	BCAL2758	Exodeoxyribonuclease VII large subunit (EC3.1.11.6), *xseA*	8.8	1.5	n.s.	n.s.				
11	G8	light blue	I35_2615	BCAL2758	Exodeoxyribonuclease VII large subunit (EC3.1.11.6), *xseA*	9.5	2.6	n.s.	n.s.				
12	E3	light blue	I35_2615	BCAL2758	Exodeoxyribonuclease VII large subunit (EC3.1.11.6), *xseA*	117.1	88.8	n.s.					
13	B8	light blue	I35_4474	bcam0580	*Cis*-2-dodecenoic acid receptor, *rpfR*	50.9	55.6	down	down	down			
14	C12	light blue	I35_4474	bcam0580	*Cis*-2-dodecenoic acid receptor, *rpfR*	47.8	36.2	down	down	down		yes	n.s.
15	F11	light blue	I35_4474	bcam0580	*Cis*-2-dodecenoic acid receptor, *rpfR*			n.s.	n.s.		yes		
16	H11	light blue	I35_4766	BCAM0853	Transposase and inactivated derivatives			down	n.s.	n.s.	yes	yes	down
17	F8	light blue	I35_4766	BCAM0853	Transposase and inactivated derivatives	27.8	3.1	down	n.s.	n.s.		yes	(down)
18	H9	light blue	I35_4766	BCAM0853	Transposase and inactivated derivatives	116.0	32.9		n.s.		yes		
19	D8	light blue	I35_4810	BCAM0897	Organic hydroperoxide resistance transcriptional regulator			down		n.s.		yes	(down)

	Clone[a]	Colour[a]	Locus ID[b]	Orthologe in J2315[c]	Description	MU_ex %wt[d]	MU_stat %wt[d]	WB[e]	Protease[e]	Biofilm[e]	BDSF synthesis[e]	AHL synthesis[e]	EPS[e]
20	D6	light blue	I35_6288/89	BCAM2393	Transcriptional regulator containing amidasedomain and AraC-type DNA-binding HTH domain/ L-serine dehydratase	117.3	63.8	n.s.					
21	C15	light blue	I35_6288/89	BCAM2393	Transcriptional regulator containing amidasedomain and AraC-type DNA-binding HTH domain/ L-serine dehydratase	112.2	67.6	n.s.					
22	G11	white	I35_6288/89	BCAM2393	Transcriptional regulator containing amidasedomain and AraC-type DNA-binding HTH domain/ L-serine dehydratase	6.9	1.7	n.s.	up				
23	*B10*	light blue	n.d.										
24	*C14*	light blue	n.d.										
25	D9	white	I35_0826	BCAM2600	Glucose-6-phosphate 1-dehydrogenase (EC1.1.1.49)/ (Transcriptional regulator, LysR	7.9	2.2	(down)	n.s.	n.s.		yes	(down)
26	H10	white	I35_2697	BCAL2840	intergenic region: pyruvate kinase, phophoglycerate kinase, *pykA*			(up)	n.s.	n.s.	yes	yes	n.s.
27	C3	white	I35_4862	BCAM0942	fimbrial PilY2	6.2	1.5	n.s.					
28	G13	white	I35_6715	BCAM2835	salicylate esterase, *estC*	7.3	9.2	(up)	n.s.	n.s.		yes	(down)
29	G15	white	I35_0633	-	Capsular polysaccharide export system protein KpsS	8.5	2.5	n.s.					

a Nomenclature according to the transposon screen; colour, colour of the clone in transposon screen.

b Nomenclature according to GenBank file (accession no. HG938370, HG938371, HG938372), two IDs are indicative for intergenic region.

c Orthologs were identified as described in the Material and Methods section.

d Results of b-galactosidase analysis. MU, Miller Units in % of wild type level; cells harvested at exponential (ex, OD600=2.5) or stationary (stat, overnight culture) growth phas

e Results of phenotypic analysis performed as described in Material and Methods. Results were compared to wild type level, brackets indicate a rather alteration, but results need to be confirmed. Empty fields, values not determined; n.s., not significantly altered; blue indicates up-, green indicates down regulation compared to the wild-type level.

4.4.2 Construction of an insertion mutant of *lepR*

In order to confirm the results obtained by analysis of the in-frame deletion mutant of *lepR*, an insertion mutant of *lepR* was constructed as described in Material and Methods.

4.4.3 Extended analysis of cepacian production controlled by LepR

Due to the abrogation of EPS synthesis observed in the *lepR* mutant H111-lepR, the individual regulation of both the *bce-I* and *bce-II* clusters was investigated. The gene *lepR* was overexpressed by introducing pBBR(lepR) into mutants of the *bce-I* or *bce-II* clusters. Deletion mutants of H111-rpfR, H111-gtaB (I35_4939, a gene of *bce-II*, corresponding to bcam1010), H111-bceC (I35_4769, a gene of *bce-I*, corresponding to bcam0855) and the triple mutant H111-rpfR-gtaB-bceC was complemented with pBBR(lepR) and inspected for EPS production (Figure S 4.1).

As with the triple mutant, that did not produce cepacian, both single mutants H111-bceC and H111-gtaB showed reduced or absent EPS production, respectively. Upon genetic complementation by *lepR*, EPS production was not restored in H111-bceC, but was partially complemented in H111-gtaB, almost reaching wild-type level. EPS production was not restored in H111-rpfR-gtaB-bceC/pBBR(lepR), confirming the necessity of both *bce-I* and *bce-II* for EPS production. Under the conditions tested, RpfR was not found to alter cepacian production in *B. cenocepacia* H111. The results indicate that i) the *bce-I* cluster is able to compensate for *bce-II* (eventually resulting in altered EPS composition) and ii) LepR (if overexpressed) is able to induce cepacian formation (via the *bce-I* cluster).

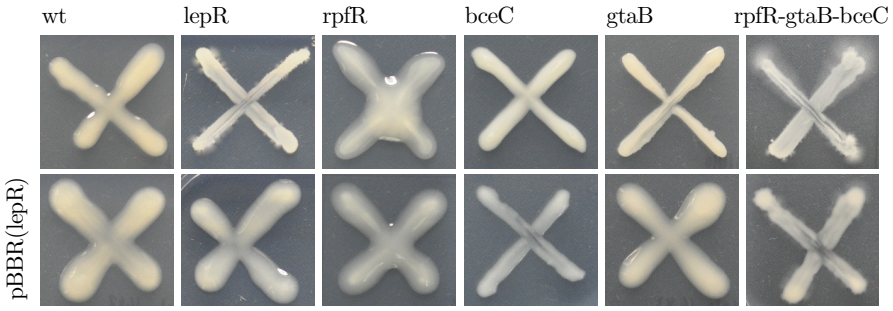

Figure S 4.1 EPS production requires both a functional BceC and LepR. EPS production was tested on YEM plates of *B. cenocepacia* H111 mutant strains (upper panel) and same strains carrying pBBR(lepR) (lower panel); wt, wildtype; *lepR*, H111-lepR; rpfR, H111-rpfR; bceC, H111-bceC; gtaB, H111-gtaB; rpfR-gtaB-bceC, H111-rpfR-gtaB-bceC. Strains were grown for 48 h at 37 °C.

4.4.4 Extended analysis of the effect of LepR on colony morphology

In order to analyse the contribution of LepR to colony morphology, *lepR* was overexpressed in *B. cenocepacia* H111ΔrpfR. RpfR was previously shown to suppress wrinkly colony morphology in *B. cenocepacia* (Elisabeth Steiner in preparation). In contrast, in H111ΔrpfR/pBBR(lepR) the wrinkly colony morphology reverted to a smoother one, suggesting that LepR positively regulates genes required for smooth colony morphology, or is involved in the repression of genes required for wrinkly colony morphology, respectively (Figure S 4.2A). Following this, the triple mutant H111-rpfR-gtaB-bceC was transformed with pBBR(lepR) and tested for colony morphology on NYG plates. Again, no wrinkly macrocolony structure was detected. This result indicates the effect seen on colony morphology is not due to increased cepacian production induced by LepR masking the wrinkly phenotype.

Gene bcam1349 (bcam1349 in J2315, I35_5200 in H111) was shown to encode a regulator of gene expression controlling wrinkly colony morphology in *B. cenocepacia* H111 ((Fazli *et al.*, 2011). Overexpression of bcam1349 in *B. cenocepacia* H111 induces a wrinkly phenotype, covering the full macrocolony. Complementation of H111-lepR with pBBR(1349) reduced the amount of wrinkling compared to H111/pBBR(1349) (Figure S 4.2B). Despite this, evidence of LepR-dependent regulation of Bcam1349 was not found in the RNA-Seq

analysis. However, I35_5180 (encoding polysaccharide export lipoprotein wza, bcam1330 in J2315) was found to be down regulated in H111-lepR compared to the wildtype. I35_5180 is part of the third EPS cluster in H111, which is required for wrinkly colony morphology (Fazli *et al.*, 2013).

In summary, we have provided evidence that LepR is involved in the regulation of colony morphology (positively and/or negatively), and potentially acts downstream of Bcam1349.

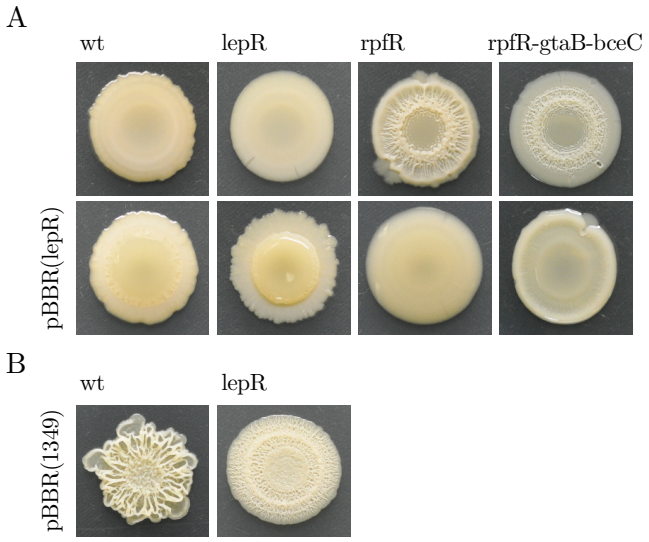

Figure S 4.2 Colony morphology is at least partially regulated by LepR. A, *B. cenocepacia* H111 mutant strains (upper panel) and the same strains carrying pBBR(lepR) (lower panel) were tested for colony morphology on NYG plates; wt, wild type; *lepR*, H111-lepR; rpfR, H111-rpfR; rpfR-gtaB-bceC, H111-rpfR-gtaB-bceC. **B**, I35_5200 (bcam1349 in J2315) was overexpressed in the wild type and the *lepR* mutant background.

4.4.5 Extended analysis of proteolytic activity controlled by LepR

It was previously shown, that *B. cenocepacia* H111 does not display proteolytic activity under elevated intracellular c-di-GMP concentrations. The vector pRpfR^{AAL}, which induces an artificially increased c-di-GMP level, was introduced into *B. cenocepacia* H111-lepR. H111-lepR pRpfR^{AAL} showed decreased proteolytic activity, similar to H111 pRpfR^{AAL} suggesting that LepR is not a downstream regulator of c-di-GMP (Figure S 4.3).

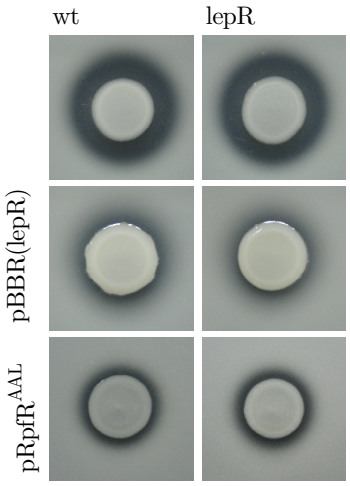

Figure S 4.3 A mutation in *lepR* does not rescue protease production upon elevated c-di-GMP level in *B. cenocepacia* H111. Proteolytic activity was tested on skim milk agar plates in *B. cenocepacia* H111 wild type (wt) or H111-lepR carrying pBBR(lepR) or pRpfR^{AAL}. Macrocolonies were grown for 48 h at 37°C.

4.4.6 Analysis of a *lepR* conditional mutant

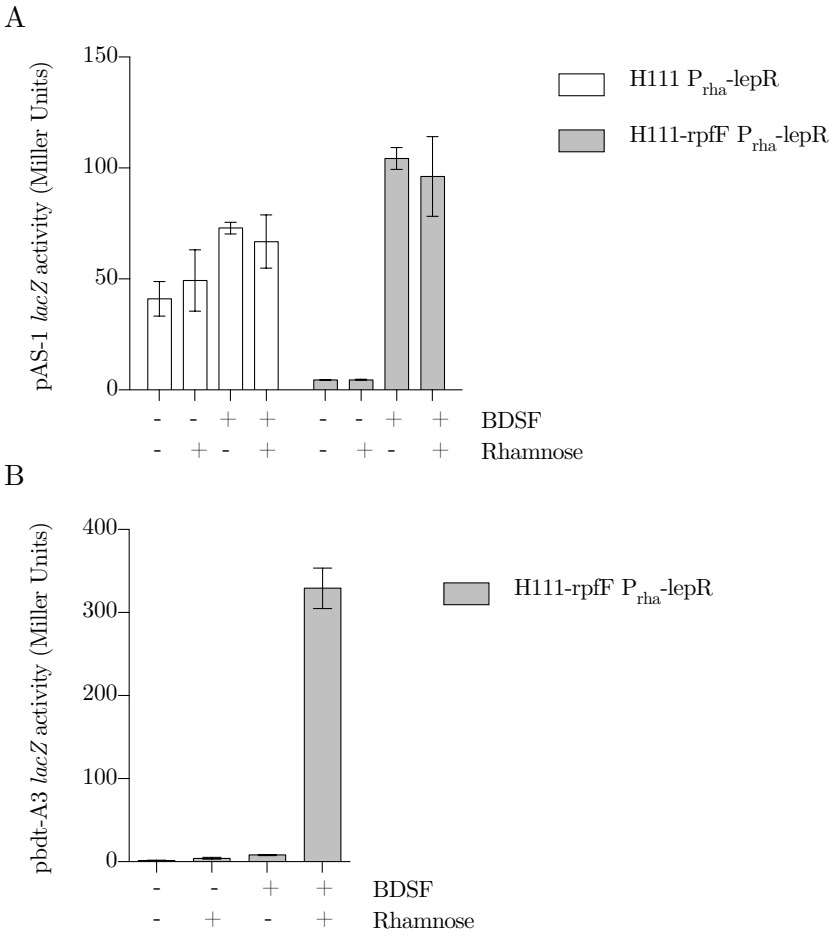

Figure S 4.4 LepR and the BDSF-dependent regulator affect *bclACB* expression via different sites in the promoter region. Strains were grown in the presence (+) or absence (-) of 0.2 % rhamnose and/ or 10 µM BDSF. **A**, *lacZ* expression from pAS-1 is independent of LepR. **B**, *lacZ* expression of pbdt-A3 is only induced in the presence of both *lepR* and BDSF. Shown are mean values with SD, n≥2.

4.4.7 A heterologous expression system to test whether LepR is a DNA-binding transcriptional regulator

A heterologous expression system was constructed in *E. coli* according to the methods of Guzman *et al.* and Schu *et al.* (Guzman *et al.*, 1995; Schu *et al.*, 2009). The gene *lepR* was cloned into pBAD24 under the control of an arabinose inducible promoter, resulting in pBAD24(lepR). Previous experiments had revealed that LepR is required for expression of *bclA*, analysed by a *lacZ* fusion in pbdt-A3. Therefore, pBAD24(lepR) was introduced into *E. coli* Top10 together with pbdt-A3. The transformed cells were tested by the addition of L-arabinose to the medium to induce *lepR* and analysed for *lacZ* expression. Had *bclA* been induced, this would have confirmed LepR as a DNA-binding transcriptional regulator. However, under the conditions tested, no significant expression of the reporter gene was detected (Figure S 4.5). The same experiment was performed with exogenous complementation of the medium by BDSF and AHLs, which might contribute to the activity of LepR. Again, no significant expression of the reporter gene was detected (data not shown).

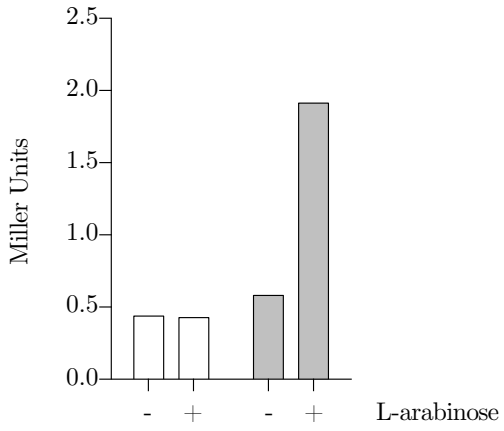

Figure S 4.5 A heterologous expression system did not show LepR to be a DNA-binding transcriptional activator. β-galactosidase activity was measured in *E. coli* Top10 carrying pBAD24(lepR) and the empty vector pbdt-lacZ (white) or pbdt-A3 (grey) in the absence or presence of 0.2 % L-arabinose, required for expression of *lepR* from pBAD24. The lectin promoter *bclACB* in pbdt-A3 was not significantly activated upon *lepR* expression. Data show one measurement.

4.4.9 LepR does not form a protein-protein complex with RpfR

Previous experiments (Elisabeth Steiner, in preparation) had suggested that the BDSF receptor RpfR modulates expression levels of other proteins by the formation of protein-protein complexes. As we observed a partial overlap of the RpfF- and LepR regulated genes, we assumed LepR to be a candidate for interaction with RpfR. In order to test this putative interaction, the bacterial two-hybrid system BACTH was applied (see Material and Methods 4.5). The system is based on the enzyme adenylate cyclase from *Bordetella pertussis*, which is functional only when its two catalytic domain fragments T25 and T18 are physically fused. The two potentially interacting proteins RpfR and LepR were genetically fused to T25 and T18 fragments in two compatible plasmids and analysed as described in Material Methods 4.5. However, no protein-protein interaction of RpfR and LepR was observed (Figure S 4.6). The same assay was performed with MacConkey agar plates containing 10 µM BDSF and by using competent *E. coli* BTH101, respectively. Again, no interaction of the two proteins was observed. All BACTH assays were considered functional, as a positive control was included and showed a positive, red macrocolony colour. However, expression of *lepR* under the conditions used was not confirmed.

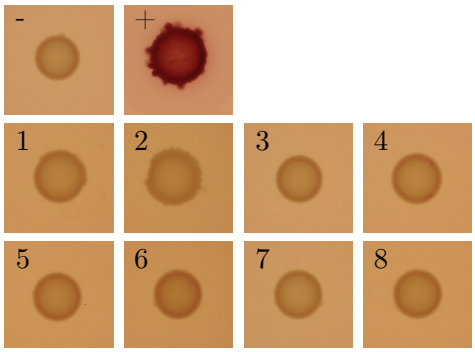

Figure S 4.6 LepR does not form a protein-protein complex with RpfR *in vitro*. LepR and RpfR was cloned in pT18 and pT25 and tested for protein-protein interaction by BACTH. A dark red colour indicates positive interaction. -, negative control; + positive control; following macrocolonies 1-8 are combinations of the pT18 or pT25 tagged RpfR and LepR in *E. coli* BTH101: 1, pUT18-rpfR and pKNT25-lepR; 2, pUT18-rpfR and pKT25-lepR; 3, pUT18C-rpfR and pKNT25-lepR; 4, pUT18C-rpfR and pKT25-lepR; 5, pKT25-rpfR and pUT18-lepR; 6, pKT25-rpfR and pUT18C-lepR; 7, pKNT25-rpfR and pUT18-lepR; 8, pKNT25-rpfR and pUTC18-lepR. Data shown are representative pictures of two replicates, after 3 days incubation on MacConkey Amp, Km plates.

4.5 Materials and Methods

Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S 4.3 or Table S 4.4, respectively. *E. coli* and *B. cenocepacia* cells were routinely grown aerobically in Luria-Bertani (LB, Difco) medium at 37 °C. Antibiotics were used at the concentrations (µg/ml) indicated in brackets: for *E. coli*, ampicillin (100), kanamycin (25), gentamicin (10), chloramphenicol (30), trimethoprim (50); and for *B. cenocepacia*, kanamycin (100), gentamicin (20), chloramphenicol (80) and trimethoprim (100).

Oligonucleotides

Oligonucleotides (Microsynth AG, Balgach) used in this study are listed in Table S 4.5.

PCR and plasmid DNA purification and gel extraction

PCR and plasmid DNA purification and gel extraction was performed with kits obtained from QIAGEN, according to the manufacturer's protocol. DNA was stored at -20 °C.

Construction of insertion mutants

An internal fragment of the gene to be interrupted was amplified from *B. cenocepacia* H111 genomic DNA by PCR using appropriate primers (for I35_4766: AS91, AS92; for I35_2688: AS93, AS94). The fragment was cloned into pGEM®-T Easy vector (Promega), the plasmid was transformed to *E. coli* DH5α and checked by sequencing. The DNA fragment was digested and ligated into the suicide vector pSHAFT2 or pEX18Gm at the NotI or EcoRI restriction site, respectively. The vector was transformed into *E. coli* CC118λpir for pSHAFT2 or *E. coli* DH5α for pEX18Gm and then delivered to *B. cenocepacia* H111 by triparental mating. Mutants were screened by colony PCR.

Construction of in-frame deletion mutants

For generation of in-frame deletion mutants, the protocol established by Flannagan *et al.* was used (Flannagan *et al.*, 2008). Briefly, for generating the *lepR* mutant, both flanking regions of the to be deleted gene fragment were PCR amplified using genomic *B. cenocepacia* H111 DNA and primers for flanking region 1: AS99 and AS100 (489bp); and for flanking

region 2: AS108 and AS135 (304bp). Joined flanking regions include 69 bp of *lepR*, with start and stop codon in-frame. Both fragments were individually cloned into the pGEM®-T Easy vector (Promega) according to the manufacturer's protocol, *E. coli* DH5α was transformed with the plasmid, and the plasmid was checked by sequencing (primers: M13_F and M13_R). Both DNA fragments were digested with restriction enzymes as follows: for flanking1 (99.100); EcoRI, XhoI and for flanking2 (108.135); XhoI, KpnI, and subcloned into vector pGPI-SceI digested with EcoRI and KpnI. Triple ligation was performed with the linearized vector pGPI-SceI and both inserts. *E. coli* SY327λpir cells were transformed with the ligation product pGPI-SceI(99.100-108.135), tested by PCR (primers: ES44/45) and sequencing. By means of triparental mating, pGPI-SceI(99.100-108.135) was introduced into *B. cenocepacia* H111 or *B. cenocepacia* H111-rpfF, respectively. Transformants were selected on PIA containing trimethoprim (100 μg/ml) and PCR tested. Following this, the I-SceI expression plasmid pDAIGm-SceI was introduced and cells were selected on PIA containing gentamicin (20 μg/ml). The generated mutant *B. cenocepacia* H111-lepR was tested by PCR profiling, using *lepR* flanking primers (AS87/ AS90, wt:1673 bp, *lepR*: 803 bp).

Construction of conditional mutants

The first 579 bp region of I35_4766 was PCR amplified with Phusion polymerase and the primers AS145 and AS146. The PCR product was subcloned into pGEM®-T Easy vector (Promega) according to the manufacturer's protocol. *E. coli* DH5α was transformed with the plasmid, and transformants were selected on LB containing ampicillin (100 μg/ml) and X-Gal. Insertion was verified by PCR (primers: M13_F and M13_R) and sequencing. The plasmid was extracted and digested with NdeI and XbaI. After purification, the fragment was inserted into the corresponding sites of pSC200 and transformed to *E. coli* CC118λpir chemically competent cells. After verifying the insert by PCR (primers: pSC200_F, AS146) the plasmid was transferred by triparental mating to *B. cenocepacia* H111 and *B. cenocepacia* H111-rpfF, following selection on trimethoprim (100 μg/ml).

Construction of plasmids for genetic complementation

Genes used for complementation studies were amplified by PCR using *B. cenocepacia* H111 genomic DNA as template and appropriate primers (for I35_4766: AS98, AS104). Amplified DNA fragments were subcloned into pGEM®-T Easy vector (Promega), according to manufacturer's protocol and *E. coli* DH5α cells were transformed with the plasmid, using

blue/ white screening on LB Amp/X-Gal. Plasmids were purified and then digested with the restriction endonucleases XbaI and HindIII. The DNA fragment was inserted into the linearized vector pBBR1MCS-5. *E. coli* DH5α cells were transformed with the vector, which was then transferred to *B. cenocepacia* H111 by triparental mating.

Transformation

5 µl DNA was added to 100 µl competent cells (prepared according to Chung *et al.* (Chung *et al.*, 1989) and incubated for 10 min on ice. Cells were then shocked for 1 min at 42 °C. The sample tube was put back on ice for 1 min after which 500 µl SOC was added. The transformed cells were incubated for 1 h at 37 °C, 225 rpm. After incubation, the cells were spread on selection plates (LB containing antibiotics and X-Gal as appropriate). Transformants were tested by PCR and, if required, by sequencing analysis.

Conjugative plasmid transfer (Triparental Mating)

To transfer a plasmid into *B. cenocepacia,* a triparental mating according to de Lorzeno and Timmis was performed (de Lorenzo and Timmis, 1994). As helper strains, *E. coli* HB101/pRK600 or *E. coli* MM294/pRK2013 was used. Briefly, 2 ml overnight culture of each donor (carrying the plasmid of interest), helper and the *Burkholderia* recipient strain, were washed twice and resuspended in 500 µl LB. 100 µl of both donor and helper, were mixed and incubated at RT for 10 min. Following this, 200 µl of the recipient strain was added. 50 µl spots of the mixture were transferred to LB plates and incubated for at least 6 h up to overnight at 37 °C. The bacteria were resuspended in 0.9 % NaCl, spread on PIA plates containing antibiotics as appropriate and incubated at 37 °C. Clones were restreaked and tested by colony PCR.

DNA sequence analysis

Samples were analysed by Sanger Sequencing at Microsynth AG, Balgach, Switzerland using appropriate primers. The sequences obtained were analysed using CLC main workbench (CLCbio). Promoter prediction was performed with BPROM (www.softberry.com). Identification of palindromic sequences was performed with EMBOSS explorer (http://emboss.bioinformatics.nl/cgi-bin/emboss/palindrome).
Analysis of protein homology was performed with Phyre2 V2.0

(http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index), the *Burkholderia* database (Winsor *et al.*, 2008) or NCBI (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi).

Promoter fusions to *lacZ* in pSU11 or pbdt-*lacZ*

Promoter regions were PCR amplified by using *B. cenocepacia* H111 genomic DNA as template, unless otherwise mentioned. For translational fusions (in pbdt-*lacZ*), primers were designed to clone the first few codons of each gene in-frame with the reporter gene *lacZ*. The PCR fragments were cloned into pGEM®-T Easy vector (Promega) according to manufacturer's protocol for sequencing. Following this, the fragments were digested and cloned into restriction sites as follows: XhoI and HindIII for pSU11 and EcoRI and NcoI for pbdt-*lacZ*. Constructs were tested by PCR using the primers AS79 and AS80, and where appropriate inserts were checked by sequencing. Plasmids were transferred into *B. cenocepacia* by means of triparental mating.

Transposon mutant screening

B. cenocepacia miniTn*5* mutant strains (in-house collection, C. Jenul) were recovered in LB containing kanamycin (100 µg/ml) for 30 min at 37 ºC, with 50 rpm shaking. Overnight cultures of the helper strain *E. coli* HB101/pRK600, the donor *E. coli* DH5α/pSU11-A1 and the recovered recipient were harvested by centrifugation, and the pellets washed with LB and resuspended in 3 ml LB. Donor and helper were mixed 1:1 and incubated for 30 min at room temperature. Following this, 3 ml recipient suspension was added and the resulting mixture was pipetted onto LB plates in 100 µl spots. Plates were incubated overnight at 37 ºC. The following day, the spots were resuspended in 0.9 % NaCl and spread on PIA plates containing trimethoprim (100 µg/ml) and X-Gal. Bacteria were incubated for 48 h at 37 ºC and then maintained at room temperature and purified on PIA containing kanamycin (50 µg/ml), trimethoprim (100 µg/ml) and X-Gal for further analysis.

Arbitrary PCR of selected clones was performed as previously described, with slight modifications (Huber *et al.*, 2002). Briefly, first round PCR was performed using colony material as the template, with the primers ARB6 (GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC, 0.1 µM) and specint (GACCTTGCCATCATGACTGTGCTG, 1 µM) with Go Taq Polymerase under the following conditions i) 5 min at 95 ºC; ii) 6x (30 sec at 95 ºC, 30 sec at 30 ºC, 1 min at 72 ºC), iii) 30x (30 sec at 95 ºC, 30 sec at 45 ºC, 1 min at 72 ºC), iv) 5 min at 72 ºC. Of the PCR

product 5 µl was used for second round PCR, using the primers ARB2 (GGCCACGCGTCGACTAGTAC, 0.1 µM)
and specext (AACGCGTATTCAGGCTGACC, 0.1 µM) with Go Taq Polymerase under the conditions i) 5 min at 95 °C; ii) 30x (30 sec at 95 °C, 30 sec at 50 °C, 1 min at 72 °C), iii) 5 min at 72 °C. PCR products were purified and submitted for sequencing using the primer arb-seq (ATGAATGTTCCGTTGCGCTG).

RNA-Seq and data analysis

Cells were grown and harvested as previously described (Schmid *et al.*, 2012). Briefly, overnight cultures were diluted 1:100 in LB (Lennox) and grown to stationary phase, prior to inoculation of 100 ml LB to give a starting OD_{600} of 0.05. Cultures were grown at 37 °C, with 200 rpm shaking to OD_{600} of 2.0-2.1 (late exponential growth phase). Following this, 13.5 ml of each culture was rapidly transferred to tubes containing 1.5 ml of stop solution (10 % phenol (buffered with 10 mM Tris-HCl, pH 8) in ethanol) and centrifuged for 6 min, 5000 rpm at 4 °C. The supernatant was discarded and the pellet frozen in liquid nitrogen.

Total RNA was extracted and analysed as described by Lardi *et al.* (Lardi *et al.*, 2015). After a successful DNAse treatment, the quality of the RNA was checked by RNA Nano Chips (Agilent 2100 Bioanalyzer; RNA Integrity Number >8).

Five hundred ng of RNA was used for first and second strand cDNA synthesis. Library preparation and purification was performed using the Encore® Complete Prokaryotic RNA-Seq DR Muliplex System (Nugen), which uses Insert Dependent Adaptor Cleavage (InDAC) technology for removal of ribosomal RNA transcripts. The cDNA library was assessed and quantified by capillary electrophoresis using the Agilent High Sensitivity D1000 Screen Tape System (Agilent). Illumina single-end sequencing was performed using the HiSeq2500 instrument at the FGCZ (University of Zurich). Sequence reads were processed and mapped to the *B. cenocepacia* H111 genome (Carlier *et al.*, 2014) using CLC Genomics Workbench v7.0 (CLC bio). Differential expression analysis was performed using the DESeq software v1.20 (Anders and Huber, 2010). Clusters of Orthologous Groups (COGs) from http://clovr.org/docs/clusters-of-orthologous-groups-cogs/ were given the following letter associations: [D], Cell cycle control, cell division, chromosome partitioning; [M], Cell wall/membrane/envelope biogenesis; [N], Cell motility; [O], Post-translational modification, protein turnover, and chaperones; [T], Signal transduction mechanisms; [U], Intracellular trafficking, secretion, and vesicular transport; [V], Defense mechanisms; [W],

Extracellular structures, [Y], Nuclear structure; [Z], Cytoskeleton; [A], RNA processing and modification; [B], Chromatin structure and dynamics; [J], Translation, ribosomal structure and biogenesis; [K], Transcription; [L], Replication, recombination and repair; [C], Energy production and conversion; [E], Amino acid transport and metabolism; [F], Nucleotide transport and metabolism; [G], Carbohydrate transport and metabolism; [H], Coenzyme transport and metabolism; [I], Lipid transport and metabolism; [P], Inorganic ion transport and metabolism; [Q], Secondary metabolites biosynthesis, transport, and catabolism; [R], General function prediction only; [S], Function unknown.

4.5.1 Phenotypic analysis

Analysis of protease activity

Bacterial overnight cultures were washed and adjusted to an OD_{600} of 1.0 in LB. Of this cell suspension, 5 µl was spotted on skim milk plates (LB Lennox, 2 % skim milk, 1 % agar) and incubated at 37 °C. Plates were inspected visually after 24 and 48 h. The assay was repeated at least three times.

Analysis of BDSF and AHL production level

Qualitative assay was performed using cross-streak experiments. These were performed as described in Suppiger *et al.* (Suppiger *et al.*, 2016a). Briefly, the sensor strain (for AHL analysis *P. putida* F117/pAS-C8; for BDSF analysis *B. cenocepacia*-rpfF/pAN-L15 (Suppiger *et al.*, 2016a)) was streaked perpendicular to the strain to be tested on LB plates (without antibiotics), incubated for 24 or 48 h, after which time plates were visually inspected for reporter gene expression.

Quantitative assay was performed using liquid culture as described in Suppiger *et al.* (Suppiger *et al.*, 2016a). Briefly, 100 µl of supernatant from an overnight culture of the test strain was mixed with 100 µl of the biosensor strain (for AHL analysis: *P. putida* F117/pAS-C8; for BDSF analysis: *B. cenocepacia*-rpfF/pAN-L15 (Suppiger *et al.*, 2016a)) and grown to exponential growth phase. After 20 h incubation at 30 °C, fluorescence (excitation at 485 nm, emission at 528 nm) or luminescence, was recorded using a microtiter plate reader (Syntergy HT; Bio-Tek, Germany).

Colony morphology

Colony morphology was determined on NYG plates (0.5 % peptone, 0.3 % yeast extract, 2 % (w/v) glycerol, 1.5 % agar). A bacterial overnight culture was washed and adjusted to an OD_{600} of 1.0 in 0.9 % NaCl solution. Of this cell suspension, 5 µl was spotted on NYG plates and these were incubated for 3 days at 37 °C, followed by at least 2 days at room temperature.

Biofilm quantification

Biofilm formation by *B. cenocepacia* H111 was quantified under static conditions in microtiter plates as described by Aguilar *et al.* (Aguilar *et al.*, 2014).

Measurement of β-galactosidase activities

β-galactosidase Activity was measured as previously described in Section 3.5.

Extraction and quantification of c-di-GMP

Bacterial overnight cultures were subcultured in LB broth, and 5 ml was harvested when this subculture had reached an OD_{600} of 2.0. C-di-GMP was extracted as previously described (Suppiger *et al.*, 2016b). Briefly, the bacterial culture was centrifuged at 5000 rpm, 5 min, 4 °C, and the supernatant was discarded. The pellet was resuspended in 300 µl ice-cold extraction solvent (acetonitrile/methanol/water; 40 /40 /20; vol/ vol/ vol) and incubated on ice for 15 min. The cell suspension was heated for 10 min to 95 °C, and then centrifuged at 13000 rpm, 10 min, 4 °C. The supernatant was transferred to 2 ml Safe Seal tubes (Sarstedt 72.695.500). This extraction step was repeated twice on each pellet with 200 µl extraction solvent at 4 °C, omitting the 95 °C heating step. The supernatants obtained for each pellet were combined and centrifuged at 13000 rpm, 10 min, 4°C, and transferred to a fresh 2 ml Safe Seal tube. Solvent was evaporated in a Speedvac at 60 °C. Quantification was performed by LC-MS/MS (Burhenne and Kaever, 2013).

EPS production

Bacteria from overnight cultures were streaked on YEM agar plates (0.05 % yeast extract, 0.4 % D-mannitol, 1.5 % agar) and incubated at 37 °C for 48 h.

Pellicle formation

An overnight culture was diluted 1:100 in NYG broth (0.5 % peptone, 0.3 % yeast extract, 2 % (w/v) glycerol) and incubated in a closed vessel without shaking at room temperature for at least 5 days. The pellicle was inspected visually, and strength was measured by counting the number of glass beads carried before the pellicle was detached (no beads held, pellicle absent; 1-4 beads held, thin pellicle present; 5 or more beads held, robust pellicle present).

Swarming motility

Swarming motility was measured on semi-solid NB plates (0.5 % peptone, 0.3 % beef extract, 0.4 % agar). Five µl of bacterial overnight culture was spot inoculated at the centre of each plate. The plate was incubated for 24 h at 30 ºC and the swarming zone was documented photographically.

Heterologous gene expression system

For construction of pBAD24(*lepR*), I35_4766 was PCR amplified with AS142 and AS139 primers using *B. cenocepacia* H111 genomic DNA as template, and cloned into pGEM-T Easy vector (Promega) prior to subcloning in pBAD24 digested by EcoRI and HindIII (Guzman *et al.*, 1995).

For construction of the heterologous expression system, competent *E. coli* Top10 cells were transformed simultaneously with both purified vectors pBAD24(*lepR*) and pbdt-A3. Cells carrying both vectors were selected on LB plates containing both ampicillin and gentamicin.

Expression analysis: *E. coli* Top10/pBAD24(*lepR*) pbdt-A3 from overnight cultures were diluted in LB containing ampicillin and gentamicin to an OD_{600} of 0.05. Cells were grown to an OD_{600} of 0.5 at 37 ºC, with 225 rpm shaking, and then supplemented with 0.2 %, 0.02 % or 0 % L-arabinose and grown for 4 h. Following this, 1 ml of the cells were harvested and *lacZ* expression was analysed by measuring β-galactosidase activity as previously described.

Protein-protein interaction

Protein-protein interaction was analysed used the BACTH system (euromedex) according to Battesti *et al.* (Battesti and Bouveret, 2012). I35_4766 (AS143 and AS144) and *rpfR* (E. Steiner) were PCR amplified, cloned into pGEM-T Easy vector (Promega), digested by XbaI

and KpnI and ligated with the linearized vectors of the BACTH system: pKT25, (N-terminal tag, KmR); pUT18C, (N-terminal tag, AmpR); pKNT25, (C-terminal tag, KmR) and pUT18, (C-terminal tag, AmpR) (euromedex). Chemically competent *E.* coli DHM1 cells were transformed with two compatible plasmids expressing either *rpfR* or I35_4766. The cells were grown on LB plates containing ampicillin and kanamycin for 48 h at 30 °C. From each transformation plate, 0.5 ml LB containing ampicillin, kanamycin and 0.5 mM IPTG was inoculated with three clones and grown overnight at 30 °C. Of each culture, 2 µl was spotted on MacConkey agar plates. The plates were incubated for at least 4 days at 30 °C and then analysed by visual inspection.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Cells were harvested from NB plates after 24 h incubation as described previously (Inhülsen *et al.*, 2012). Bacterial pellets were resuspended in 300 µl sample buffer (4x: 4 ml 20% SDS, 4 ml glycerol, 2 ml Tris/HCl pH 6.8, 0.4 % bromophenol blue and β-Mercaptoenthanol 1:100 in 1x buffer). Sample was boiled 10 min at 100 °C.

For SDS-PAGE, a 12% resolving gel was routinely prepared and covered with a 5 % stacking gel. Electrophoresis was performed in running buffer using Mini-PROTEAN® Tetra System (Bio-Rad), with Page Ruler Plus Prestained Protein Ladder (Thermo Scientific 26619) as the protein standard. The gel was stained for 5 min in staining solution and destained until bands were visible.

Resolving gel 12%: 6.78 ml dH_2O, 8 ml Acryl-bis 30 %, 5 ml Buffer 4x pH 8.8), 200 µl APS 10 %, 20 µl TEMED

Stacking gel: 2.78 ml dH_2O, 0.83 ml Acryl-bis 30%, 1.25 ml Buffer 4x pH 6.8, 50 µl APS 10%, 5 µl TEMED

Buffer 4x, pH 8.8: 90.83 g Trizma Base, 2 g SDS, dH_2O to a total volume of 500 ml, pH 8.8

Buffer 4x, pH 6.8: 30.28 g Trizma Base, 2 g SDS, dH_2O to a total volume of 500 ml, pH 6.8

Running Buffer, 1x: 3 g Trizma Base, 14.4 g Glycine, 1 g SDS, dH_2O to a total volume of 1000 ml

Staining solution: 1 g R-250, 50 % Methanol, 10 % acetic acid, dH_2O to a total volume of 1000 ml

Destaining solution: 40 % Methanol, 10 % acetic acid in dH_2O

Western Blot Analysis

For the detection of proteins separated by SDS-PAGE, a western blot analysis was performed. After electrophoresis, the gel was blotted onto a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond™-P, GE-Healthcare) using the Bio Rad Trans-Blot®Turbo™ Transfer System (Transfer buffer: 5.8 g Trizma Base, 2.9 g Glycine, 0.37 g SDS, 200 ml Methanol, to a total volume of dH_2O 1000 ml, pH 8.3). The membrane was washed twice in TBS (10 mM, pH 7.5 Tris/HCl, 50 mM NaCl) for 10 min, incubated for 1 h at room temperature in blocking buffer (3 g BSA in 100 ml TBS), washed twice in TBS-T (20 mM, pH 7.5 Tris/HCl, 500 mM NaCl, 0.5 % (v/v) Tween20, 0.2 % (v/v) Triton X-100). The membrane was incubated for 1 h in binding buffer (Blocking buffer:TBS, 1:1) containing primary antibody (1:3500, rabbit α-BclB or rabbit α-AidA). The membrane was then washed twice with TBS-T, and once with TBS and incubated with HRP-conjugated secondary antibody (goat-α-rabbit HRP in blocking solution; 1:10000) for at least 1 h. The membrane was washed four times for 5 min in TBS-T. Sample detection was performed using the chemiluminescence detection Kit (ECL) and visualised using BioRad Quantity One (1-D Analysis Software 4.6.3).

Supporting Tables S 4.2-S 4.5

Table S 4.2 LepR is a global regulator in *B. cenocepacia* H111. Genes that are differentially expressed in the *lepR* mutant or the complemented mutant relative to the wild type with a fold change ≥4 are shown, as well as *zmpA* and *rsaM,* which are regulated in response to LepR but with a lesser fold change. Fold changes with a significance of p <0.12 (DESeq2 analysis) are shown in bold. Gene names that are differentially expressed in both the *lepR* and the *rpfF* mutants are shown in bold.

Class[a]	Locus ID[b]	Gene[b,c]	Ortholog in J2315[d]	Description[b]	FC (1683/wt)[e]	FC (comp/wt)[e]
E	I35_0017	I35_0017	BCAL0015	Branched-chain amino acid transport ATP-binding protein LivG (TC 3.A.1.4.1)	0.90	**0.09**
E	I35_0018	**I35_0018**	BCAL0016	Branched-chain amino acid transport ATP-binding protein LivF (TC 3.A.1.4.1)	0.79	**0.07**
E	I35_0019	**I35_0019**	BCAL0017	Leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein	0.99	**0.04**
E	I35_0022	I35_0022	BCAL0020	Leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein	0.90	**0.20**
C	I35_0032	atpB	BCAL0030	ATP synthase A chain	1.03	**4.68**
C	I35_0033	**atpE**	BCAL0031	ATP synthase C chain	0.85	**5.16**
C	I35_0034	atpF	BCAL0032	ATP synthase B chain	0.76	**7.04**
C	I35_0035	**atpH**	BCAL0033	ATP synthase delta chain	0.95	**7.97**
	I35_0059	I35_0059	BCAL0057	Small-conductance mechanosensitive channel	0.60	**0.08**
	I35_0072	I35_0072		virulence associated protein C	**0.08**	0.46
	I35_0151	**fliA**	BCAL0144	RNA polymerase sigma factor for flagellar operon	0.71	**0.06**
	I35_0189	**I35_0189**	BCAL0199	Autotransporter adhesin	1.27	**0.07**
	I35_0190	**I35_0190**	BCAL0200	Autotransporter adhesin	1.01	**0.19**
H	I35_0198	hppD	BCAL0207	4-hydroxyphenylpyruvate dioxygenase	0.87	**0.09**
J	I35_0212	**rplK**	BCAL0222	LSU ribosomal protein L11p (L12e)	1.28	**6.64**
J	I35_0219	**rpsL**	BCAL0229	SSU ribosomal protein S12p (S23e)	0.95	**4.03**
J	I35_0220	**rpsG**	BCAL0230	SSU ribosomal protein S7p (S5e)	1.38	**6.95**
	I35_0223	**rpsJ**	BCAL0233	SSU ribosomal protein S10p (S20e)	2.09	**6.13**
J	I35_0224	rplC	BCAL0234	LSU ribosomal protein L3p (L3e)	0.57	**5.22**
J	I35_0225	**rplD**	BCAL0235a	LSU ribosomal protein L4p (L1e)	**2.61**	**10.13**
J	I35_0226	**rplW**	BCAL0236	LSU ribosomal protein L23p (L23Ae)	0.98	**8.23**
J	I35_0227	**rplB**	BCAL0237	LSU ribosomal protein L2p (L8e)	**1.74**	**7.31**
J	I35_0231	**rplP**	BCAL0241	LSU ribosomal protein L16p (L10e)	0.76	**4.44**
J	I35_0232	rpmC	BCAL0242	LSU ribosomal protein L29p (L35e)	0.97	**4.30**
J	I35_0236	**rplE**	BCAL0246	LSU ribosomal protein L5p (L11e)	1.39	**15.52**
J	I35_0238	**rpsH**	BCAL0248	SSU ribosomal protein S8p (S15Ae)	1.38	**9.83**
J	I35_0239	**rplF**	BCAL0249	LSU ribosomal protein L6p (L9e)	1.07	**9.31**
J	I35_0240	**rplR**	BCAL0250	LSU ribosomal protein L18p (L5e)	1.10	**5.99**
J	I35_0241	**rpsE**	BCAL0251	SSU ribosomal protein S5p (S2e)	0.88	**4.53**
M	I35_0276	I35_0276	BCAL0287	Outer membrane protein W precursor	1.13	**0.19**
E	I35_0278	gltI	BCAL0289	Glutamate synthase [NADPH] large chain	**1.46**	**0.15**
	I35_0433	**rplU**	BCAL3442	LSU ribosomal protein L21p	1.96	**6.54**
E	I35_0462	aroE_2	BCAL3413	Shikimate 5-dehydrogenase I alpha	**10.49**	6.93
E	I35_0516	I35_0516	BCAL3359	NADP-specific glutamate dehydrogenase	0.78	**16.13**
J	I35_0527	rplM	BCAL3348	LSU ribosomal protein L13p (L13Ae)	0.67	**4.73**
G	I35_0533	I35_0533	BCAL3342	Phosphoglycerate mutase	**0.00**	0.84
	I35_0572	yajC	BCAL3305	Preprotein translocase subunit YajC	1.26	**4.81**
	I35_0669	I35_0669		hypothetical protein	0.78	**0**
C	I35_0670	**I35_0670**	BCAL3187	2-polyprenyl-6-methoxyphenol hydroxylase andrelated FAD-dependent oxidoreductases	1.49	**0.08**

M	I35_0755	galE	BCAL3117	UDP-glucose 4-epimerase	0.96	**4.15**
M	I35_0757	wbxA	BCAL3115	Glycosyl transferase, family 2	0.79	**6.68**
M	I35_0758	wzx	BCAL3114	Membrane protein involved in the export of O-antigen and teichoic acid	1.06	**8.89**
H	I35_0780	hemN	BCAL3094	Coproporphyrinogen III oxidase	1.14	**10.58**
G	I35_0825	pgl	BCAL3043	6-phosphogluconolactonase	0.93	**0.16**
G	I35_0826	zwf_2	BCAL3042	Glucose-6-phosphate 1-dehydrogenase	0.90	**0.21**
G	I35_0827	**malE**	BCAL3041	ABC-type sugar transport system, periplasmic component	1.43	**0.20**
G	I35_0828	**I35_0828**	BCAL3040	Permease of ABC sugar transporter	0.99	**0.15**
R	I35_0830	**I35_0830**	BCAL3038	ABC-type sugar transport systems, ATPase components	1.33	**0.23**
U	I35_1001	lepB	BCAL2866	Signal peptidase I	1.27	**4.58**
R	I35_1026	I35_1026	BCAL1034	Cytochrome oxidase biogenesis protein Sco1/SenC/PrrC, putative copper metallochaperone	0.74	**4.75**
F	I35_1133	I35_1133	BCAL1220	Cytosine/purine/uracil/thiamine/allantoin permease family protein	1.14	**0.08**
P	I35_1330	**I35_1330**	BCAL1432	Inositol transport system ATP-binding protein	1.17	**0.00**
	I35_1344	I35_1344	BCAL1446	hypothetical protein	**8.12**	4.59
S	I35_1418	I35_1418	BCAL1520	Uncharacterized protein conserved in bacteria	1.19	**0.08**
	I35_1437	I35_1437		hypothetical protein	0.70	**0.10**
S	I35_1438	I35_1438	BCAL1540	Superfamily I DNA and RNA helicases	1.16	**0.14**
I	I35_1439	I35_1439	BCAL1541	Long-chain-fatty-acid--CoA ligase	1.00	**0.15**
G	I35_1445	I35_1445	BCAL1547	Fructokinase	1.09	**0.24**
	I35_1447	I35_1447	BCAL1549	Ribose ABC transport system, permease proteinRbsC (TC 3.A.1.2.1)	1.25	**0.18**
	I35_1448	I35_1448	BCAL1550	Ribose ABC transport system, ATP-binding protein RbsA (TC 3.A.1.2.1)	1.10	**0.14**
	I35_1449	I35_1449	BCAL1551	transcriptional regulator/sugar kinase	1.20	**0.09**
E	I35_1458	I35_1458	BCAL1610	Cystine-binding periplasmic protein precursor	**0.00**	1.61
	I35_1497	I35_1497		hypothetical protein	0.66	**0**
	I35_1611	orbA	BCAL1700	Outer-membrane receptor for ferri-ornibactin	**0.10**	0.38
	I35_1691	I35_1691	BCAL1777	TonB-dependent receptor	0.68	**6.75**
	I35_1723	I35_1723	BCAL1808	putative membrane protein YeiH	0.57	**0**
S	I35_1778	pbhF	BCAL1860	PhbF	**0.39**	**0.09**
	I35_1786	I35_1786	BCAL1868	ATPase involved in DNA repair	**0.59**	**0.22**
F	I35_1806	ndk	BCAL1887	Nucleoside diphosphate kinase	1.10	**4.39**
E	I35_1846	**hom**	BCAL1926	Homoserine dehydrogenase	1.36	**5.22**
	I35_1896	**I35_1896**	BCAL1974	Glycogen debranching enzyme	0.95	**0**
T	I35_2046	**I35_2046**	BCAL2119	UspA	**0.00**	0.39
	I35_2058	I35_2058	BCAL2131	Oligopeptide transport system permease protein OppB (TC 3.A.1.5.1)	0.84	**0**
T	I35_2066	I35_2066	BCAL2138	Signal transduction histidine kinase	**0.00**	0.61
E	I35_2074	**ask**	BCAL2146	Aspartokinase	1.39	**10.40**
	I35_2077	I35_2077	BCAL2149	HhH-GPD base excision DNA repair family protein; putative 3-methyladenine DNA glycosylase/8-oxoguanine DNA glycosylase	**0.23**	0.51
E	I35_2172	hutU	BCAL2244	Urocanate hydratase	1.09	**0.20**
E	I35_2174	hutH	BCAL2246	Histidine ammonia-lyase	1.16	**0.20**
	I35_2245	I35_2245	BCAL2321	Glutathione S-transferase N-terminal domain protein	0.51	**0.00**
	I35_2273	**rpsO**	BCAL2349	SSU ribosomal protein S15p (S13e)	1.67	**4.79**
H	I35_2318	I35_2318	BCAL2391	Nicotinate-nucleotide adenylyltransferase	**0.16**	1.00
	I35_2362	I35_2362	BCAL2435	Na+/solute symporter	0.85	**0.14**
M	I35_2476	**I35_2476**	BCAL2615	Outer membrane protein (porin)	0.79	**0.07**
P	I35_2543	**cysH**	BCAL2683	Phosphoadenylyl-sulfate reductase [thioredoxin] / Adenylyl-sulfate reductase [thioredoxin]	37.48	**379.17**
S	I35_2544	I35_2544	BCAL2684	Oxidoreductase probably involved in sulfite reduction	1.87	**43.56**
J	I35_2573	**rpmB**	BCAL2714	LSU ribosomal protein L28p	**1.68**	**7.93**

	I35_2622	I35_2622		SSU ribosomal protein S20p	1.86	**7.84**
L	I35_2735	rhlE_3	BCAL0933	ATP-dependent RNA helicase RhlE	0.74	**4.15**
K	I35_2785	**I35_2785**	BCAL0883	putative transcriptional regulator for fatty acid degradation FadP, TetR family	**4.67**	3.21
G	I35_2893	I35_2893	BCAL0780	Phosphoenolpyruvate-protein phosphotransferase of PTS system / PTS system, glucose-specific IIA component	1.59	**0.06**
M	I35_2894	I35_2894	BCAL0779	Glucosamine-6-phosphate deaminase	**1.95**	**0.05**
	I35_2898	I35_2898	BCAL0769	DNA polymerase-like protein PA0670	**6.85**	8.72
K	I35_3171	slmA	BCAL0494	transcriptional regulator, TetR family	1.43	**0**
	I35_3280	I35_3280	BCAL3500	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	0.78	**0.14**
T	I35_4028	I35_4028	BCAM0028	hypothetical protein	0.76	**0.08**
	I35_4032	I35_4032	BCAM0032	hypothetical protein	0.99	**0**
	I35_4056	I35_4056	BCAM0056	transcriptional regulator, LysR-family	0.76	**0.23**
F	I35_4060	pcaB	BCAM0060	3-carboxy-cis,cis-muconate cycloisomerase	1.07	**0**
	I35_4076	I35_4076		hypothetical protein	0.70	**0.21**
	I35_4096	I35_4096	BCAM0087	hypothetical protein	0.91	**18.57**
	I35_4146	I35_4146	BCAM0165	hypothetical protein	2.00	**8.65**
S	I35_4182	**bclB**	BCAM0184	lectin	**0.16**	**5.80**
S	I35_4183	**bclC**	BCAM0185	lectin	**0.04**	**7.30**
	I35_4184	**bclA**	BCAM0186	lectin	**0.09**	**5.67**
	I35_4255	**I35_4255**	BCAM0371	putative periplasmic protein	0.52	**0**
E	I35_4317	I35_4317	BCAM0409	Phosphoserine phosphatase	**22.49**	12.41
	I35_4320	I35_4320	BCAM0412	transcriptional regulators of sugar metabolism	**0.10**	0.48
	I35_4325	I35_4325	BCAM0416	Agmatinase	0.32	**0**
	I35_4419	I35_4419	BCAM0529	Epoxide hydrolase	0.48	**0**
	I35_4461	I35_4461	BCAM0566	transcriptional regulator, LysR family	**37.48**	23.78
	I35_4614	I35_4614	BCAM0716	hypothetical protein	**0.05**	0.13
	I35_4665	I35_4665	BCAM0766	Ribose ABC transporter, periplasmic ribose-binding protein RbsB (TC 3.A.1.2.1)	0.78	**0.10**
N	I35_4676	**motB_2**	BCAM0778	Flagellar motor rotation protein MotB	0.98	**0.20**
	I35_4766	**I35_4766**	BCAM0853	transposase and inactivated derivatives	**0.00**	**23.08**
	I35_4767	**bceA**	BCAM0854	Mannose-1-phosphate guanylyltransferase (GDP) ; Mannose-6-phosphate isomerase	0.52	**26.03**
	I35_4768	bceB		Undecaprenyl-phosphate galactosephosphotransferase	0.09	**11.38**
M	I35_4769	bceC	BCAM0855	UDP-glucose dehydrogenase	0.14	**16.08**
	I35_4770	**bceD**	BCAM0857	protein-tyrosine-phosphatase	0.20	**17.11**
	I35_4771	**bceE**	BCAM0858	Polysaccharide export lipoprotein	0.55	**65.86**
D	I35_4772	**bceF**	BCAM0859	Tyrosine-protein kinase	0.31	**55.00**
	I35_4774	**bceH**	BCAM0861	Glycosyltransferase	0.81	**41.75**
	I35_4777	**bceK**	BCAM0864	Glycosyltransferase	0.53	**14.21**
	I35_4778	**bceL**	BCAM0865	Permeases of the major facilitator superfamily	0.26	**12.47**
	I35_4918	I35_4918	BCAM0996	DedD protein	1.70	**4.27**
	I35_4922	**I35_4922**	BCAM1003	Nucleoside-diphosphate-sugar epimerases	2.70	**23.39**
	I35_4923	**gca**	BCAM1004	GDP-mannose 4,6 dehydratase	0.84	**32.27**
	I35_4924	**I35_4924**	BCAM1005	O-antigen acetylase	2.46	**24.02**
	I35_4925	**I35_4925**	BCAM1006	hypothetical protein	5.62	**1253.44**
	I35_4926	I35_4926	BCAM1007	Membrane protein involved in the export of O-antigen and teichoic acid	1.16	**21.79**
	I35_4927	**I35_4927**	BCAM1008	Glycosyltransferase	0.19	**28.19**
	I35_4928	**I35_4928**	BCAM1009	O-antigen acetylase	0.20	**49.91**
M	I35_4929	**gtaB**	BCAM1010	UTP--glucose-1-phosphate uridylyltransferase	**0.09**	**41.61**
	I35_4930	**I35_4930**	BCAM1011	O-antigen acetylase	36.54	**505.56**
	I35_4972	I35_4972	BCAM1128	Glycosyl transferase, family 2	0.43	**0.13**
	I35_4975	**I35_4975**	BCAM1131	transcriptional regulator	**10.52**	3.40
P	I35_5004	**I35_5004**	BCAM1158	Arylsulfatase	**0.11**	0.17
K	I35_5015	I35_5015	BCAM1169	transcriptional regulator, IclR family	**0.13**	**0**
	I35_5089	I35_5089	BCAM1241	O-antigen acetylase	1.06	**17.19**
M	I35_5180	**I35_5180**	BCAM1330	Polysaccharide export lipoprotein Wza	**0.00**	1.02
	I35_5212	I35_5212	BCAM1360	putative dehydrogenase	**0.00**	0.96

P	I35_5231	I35_5231	BCAM1379	ABC-type Fe3+ transport system	0.77	**0.05**
S	I35_5316	I35_5316	BCAM1461	hypothetical protein	0.92	**0.09**
I	I35_5317	I35_5317	BCAM1462	short-chain dehydrogenase/reductase SDR	1.27	**0.07**
	I35_5318	I35_5318	BCAM1463	putative regulator PutR for proline utilization	0.78	**0.22**
E	I35_5341	I35_5341	BCAM1487	Leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein	1.59	**0.16**
	I35_5358	**I35_5358**	BCAM1505	Two-component hybrid sensor and regulator	**27.80**	10.08
	I35_5428	**I35_5428**	BCAM1572	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)	1.03	**0.16**
	I35_5652	I35_5652		Outer membrane usher protein	**0.01**	0.92
	I35_5709	I35_5709	BCAM1830	hypothetical protein	0.65	**0**
	I35_5746	rsaM	BCAM1869	hypothetical protein	**3.42**	1.43
	I35_5785	I35_5785		putative membrane protein YPO1482	1.47	**7.04**
S	I35_5857	**I35_5857**	BCAM2021	Methyl-accepting chemotaxis protein	0.81	**0.03**
	I35_5874	I35_5874	BCAM2039	Two-component response regulator	0.98	**0.01**
	I35_5879	I35_5879		hypothetical protein	0.47	**11.63**
E	I35_5880	**I35_5880**	BCAM2044	Asparagine synthetase [glutamine-hydrolyzing]	0.29	**63.38**
	I35_6064	I35_6064	BCAM2180	hypothetical protein	**79.64**	22.49
	I35_6065	I35_6065	BCAM2181	hypothetical protein	0.29	**0**
	I35_6079	I35_6079	BCAM2195	Acetyl-coenzyme A synthetase	1.12	**0.10**
	I35_6080	I35_6080		hypothetical protein	0.68	**0**
	I35_6087	**I35_6087**	BCAM2201	Xaa-Pro aminopeptidase	0.79	**0.14**
	I35_6144	I35_6144		Rhs family protein	0.73	**0.07**
	I35_6270	**I35_6270**	BCAM2374	Methyl-accepting chemotaxis protein	1.07	**0.02**
	I35_6272	**I35_6272**	BCAM2376	Lipoprotein releasing system transmembrane protein LolE	1.05	**0.09**
	I35_6290	**I35_6290**	BCAM2394	transcriptional regulator, GntR family	0.85	**0.14**
	I35_6291	**I35_6291**	BCAM2395	3-hydroxyisobutyrate dehydrogenase	0.76	**0.15**
	I35_6292	**I35_6292**	BCAM2396	putative pyridoxine biosynthesis protein (probably from glycolaldehide)	1.52	**0.19**
I	I35_6330	**I35_6330**	BCAM2430	Methylcrotonyl-CoA carboxylase biotin-containing subunit	1.33	**0.21**
	I35_6331	I35_6331	BCAM2431	Methylglutaconyl-CoA hydratase	**1.37**	**0.24**
I/Q	I35_6332	**I35_6332**	BCAM2432	Methylcrotonyl-CoA carboxylase carboxyl transferase subunit	**1.60**	**0.22**
I	I35_6333	**I35_6333**	BCAM2433	Acyl-CoA dehydrogenase(EC:1.3.99.10)	0.85	**0.21**
	I35_6344	I35_6344	BCAM2444	Conserved domain protein	**0.54**	**13.68**
	I35_6401	aroE_1		Quinate/shikimate 5-dehydrogenase I delta	0.67	**0.15**
	I35_6462	**I35_6462**	BCAM2565	Methyltransferase	**0.16**	0.99
	I35_6491	**I35_6491**	BCAM2593	transcriptional regulator, TetR family	0.33	**0.00**
S	I35_6644	**I35_6644**	BCAM2765	hypothetical protein	**0.00**	0.52
	I35_7010	**I35_7010**	BCAS0011	Permeases of the drug/metabolite transporter (DMT) superfamily	0.33	**0**
	I35_7055	I35_7055	BCAS0058	Fe-S protein, homolog of lactate dehydrogenase SO1521	0.90	**0.15**
	I35_7081	I35_7081	BCAS0084	transcriptional regulator, TetR family	0.63	**0.01**
	I35_7149	I35_7149		hypothetical protein	0.88	**0.25**
	I35_7158	I35_7158		RND multidrug efflux transporter	0.58	**0**
P	I35_7257	I35_7257	BCAS0240	Glutamate Aspartate periplasmic binding protein precursor GltI (TC 3.A.1.3.4)	0.98	**0**
T	I35_7278	**I35_7278**	BCAS0263	Response regulator	0.99	**0.06**
	I35_7279	**I35_7279**	BCAS0264	Histidine kinase	0.58	**0**
	I35_7343	I35_7343	BCAS0325	putative ABC transporter, periplasmic solute-binding protein SMa2305	0.63	**0**
	I35_7447	zmpA	BCAS0409	zinc metalloprotease ZmpA	**1.29**	**0.33**
	I35_7648	I35_7648		transcriptional regulator	**0.07**	0.35
	I35_7850	I35_7850	BCAS0727	Vanillate O-demethylase oxygenase subunit	1.24	**0.22**
F/H	I35_7855	I35_7855	BCAS0732	putative pyrimidine permease	**0.00**	0.96
I	I35_7862	I35_7862	BCAS0739	Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases	**2.05**	**0.06**

[a] Class according to Clusters of Orthologous Groups (COGs) as described in Material and Methods.

[b] Nomenclature according to GenBank file (accession no. HG938370, HG938371, and HG938372).

[c] in bold: rpfF$_{Bc}$ regulated (Schmid *et al.*, 2012).

[d] Orthologs were identified as described in Material and Methods.

[e] in bold: significant p-values <0.12.

Table S 4.3 Bacterial strains and plasmids used in this study.

Strains	Characteristics	Source/ Reference
Burkholderia cenocepacia		
H111	CF isolate from Germany, genomovar III	(Huber *et al.*, 2001; Carlier *et al.*, 2014)
H111-rpfF$_{Bc}$	*rpfF$_{Bc}$*::pSHAFT2 mutant of H111, Cm^R	(Schmid *et al.*, 2012)
H111-rpoN$_{Bc}$	*rpoN*Bc::pSHAFT2 mutant of H111; Cm^R	(Lardi *et al.*, 2015)
H111ΔcepI	Δ*cepI deletion* mutant of H111, markerless	(Schmid *et al.*, 2012)
H111ΔcepI rpfF$_{Bc}$	Δ*cepI* and *rpfF$_{Bc}$*::pSHAFT double mutant, Cm^R	(Schmid *et al.*, 2012)
H111-cepR	*cepR*::Km mutant of H111, Km^R	(Huber *et al.*, 2003)
H111-rpfR	in-frame deletion *rpfR* mutant of H111, markerless	E. Steiner, laboratory collection
H111-bceC	in-frame deletion *bcam0855* mutant of H111, markerless	E. Steiner, laboratory collection
H111-lepR	in-frame deletion I35_4766 mutant of H111, markerless	This study
H111-rpfF$_{Bc}$/pAN-L15	*cis*-2 fatty acid specific biosensor, Cm^R, Km^R	(Suppiger *et al.*, 2016a)
H111 P$_{rha}$-lepR	H111 expressing I35_4766 from a rhamnose-inducible promoter	This study
H111-rpfF$_{Bc}$ P$_{rha}$-lepR	rpfF$_{Bc}$::pSHAFT mutant, expressing I35_4766 from a rhamnose-inducible promoter	This study
Escherichia coli		
DH5α	F$^-$ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *recA1 endA gyrA96 thi-1 hsdR17 supE44 relAl deoR(U169)*	(Hanahan, 1983)
Top10	Δ*lacX74 ara*Δ*139*Δ*(ara-leu)*	Invitrogen
CC118λpir		(Herrero *et al.*, 1990)
SY327λpir	wild type strain; araD, Δ(lac pro) argE(Am) recA56 rif^R nalA λpir	
DHM1	non-reverting adenylate cyclase deficient (*cya*) reporter strain	Euromedex
BTH101	non-reverting adenylate cyclase deficient (*cya*) reporter strain	Euromedex
Pseudomonas putida		
F117/pAS-C8	*P.putida*, ppuI contining AHL sensor plasmid pAS-C8	(Steidle *et al.*, 2001)

Table S 4.4 List of plasmids used in this study.

Plasmid	Characteristics	Source/ Reference
pBBR1MCS-5	Broad-host-range cloning vector, Gm^R	(Kovach *et al.*, 1995)
pBBR(lepR)	pBBR1MCS-5 carrying I35_4766	This study
pbdt-*lacZ*	translational *lacZ* fusion vector, Gm^R	E. Steiner, laboratory collection
pbdt-A3	pbdt-*lacZ* containing the 172 bp (AS132-133) upstream region of *bclA*	This study
pbdt-lepR	pbdt-*lacZ* containing the promoter region of I35_4766	This study
pAS-1	pbdt-*lacZ* containing an artificial lac promoter site and the native 5'UTR of *bclA*	This study
pSU11Tp	pSU11 derivative promoter probe vector, harboring dhfr cassette from pRN3, Tp^R	(Schmid *et al.*, 2012)
pSU11 PbclA-lacZ	pSU11 containing the predicted *bclA* promoter region	(Inhülsen *et al.*, 2012)
pGPI-SceI	Suicide plasmid vector with I-SceI restriction site, Tp^R	E. Steiner, laboratory collection
pGPI-SceI (99.100-108.135)	pGPI-SceI based I35_4766 deletion plasmid, Tp^R	This study
pDAIGm-SceI	Plasmid encoding the I-SceI nuclease gene, Gm^R	E. Steiner, laboratory collection
pRK2013	Helper plasmid; RK2 derivative, *mob*$^+$ *tra*$^+$ *ori* ColE1; Km^R	(Figurski and Helinski, 1979)
pSC200	for driving expression of a targeted gene using the rhamnose-inducible *PrhaB* promoter, Tp^R	(Ortega *et al.*, 2007)
pSC(lepR)	pSC200 carrying an internal fragment of I35_4766 for insertional mutagenesis, Tp^R	This study
pEX18Gm	*oriT*$^+$ *sacB*$^+$; pUC18 MCS, gene replacement vector; Gm^R	(Hoang *et al.*, 1998)
pGEMT-easy	cloning vector for PCR products; Amp^R	Promega
pSHAFT2	Broad-host-range suicide plasmid, mobilisable for conjugation; CmR	S Shastri and M:S: Thomas, manuscript in preparation
pKNT25	BACTH system, expressing T25 fusion, for C-terminal fusion, Km^R	Euromedex
pKT25	BACTH system, expressing T25 fusion, for N-terminal fusion, Km^R	Euromedex
pUT18	BACTH system, expressing T18 fusion, for N-terminal fusion, Amp^R	Euromedex
pUT18C	BACTH system, expressing T18 fusion, for C-terminal fusion, Amp^R	Euromedex
pT25-lepR	I35_4766 fused to pKNT25 or pKT25, Km^R	This study
pT18-lepR	I35_4766 fused to pUT18 or pUT18C, Amp^R	This study
pBBR(1349)	pBBR1MCS-5 carrying I35_5200 (bcam1349 in J2315)	E. Steiner, laboratory collection
$pRpfR^{AAL}$	pBBR1MCS-5 carrying rpfR with mutated EAL domain, Gm^R	N. Schmid, laboratory collection
pBAD24	for driving expression of a targeted gene using the arabinose-inducible Pbad promoter, Amp^R	(Guzman *et al.*, 1995)
pBAD24(lepR)	pBAD24 carrying I35_4766 for heterologous expression, Amp^R	This study

Antibiotic-resistance of strains or plasmids: ampicillin (Amp^R), chloramphenicol (Cm^R), gentamicin (Gm^R), kanamycin (Km^R) and trimethoprim (Tp^R).

Table S 4.5 List of oligonucleotides used in this study.

Primer	5'>3' Sequence	Application
AS99_F	ACGTGAATTCACCGATTTTCGGAGGCTATT	in-frame deletion mutant of I35_4766_fl1
AS100_R	ACGTCTCGAGTGAAAGGCGAAACCACTCTT	in-frame deletion mutant of I35_4766_fl1
AS135_F	ACGTCTCGAGaCGTATCGCGTCGGTTGAT	in-frame deletion mutant of I35_4766_fl2
AS108_R	ACGTGGTACCTGTCCGAAAACGAATCGAC	in-frame deletion mutant of I35_4766_fl2
AS98_F	AAG CTTACCGATTTTCGGAGGCTATT	for pBBR complementation of I35_4766 pBBR(lepR)
AS104_R	TTCTAGAACGGACATGATGGAAAAACC	for pBBR complementation of I35_4766 pBBR(lepR)
AS136_F	ACGTGAATTCTAACAAGCGTGACGGATTTG	promoter region I35_4766 for cloning in pbdt-*lacZ*
AS137_R	ACGTCCATGGaTGAAAGGCGAAACCACTCTT	promoter region I35_4766 for cloning in pbdt-*lacZ*
AS143_F	GCTTCTAGAGTTCTTCGATGAGCTTAACGAT	to clone I35_4766 into pT25 and p18 vectors of the BACTH system
AS144_R	TAGCGGTACCCAACCGACGCGATACGCGTATTC	to clone I35_4766 into pT25 and p18 vectors of the BACTH system
AS148_F	GCTCCTGCAGCCACGGTGTGCGTCCATG	to amplify P*lac* of pBBR1MCS-5 to create pAS-1
AS149_R	CTCGGGTACCCACACAACATACGAGC	to amplify P*lac* of pBBR1MCS-5 to create pAS-1
AS150_F	GCTCGGTACCTTTCGTCATGCTCGGTACG	to amplify 5'UTR of P*bclA* to create pAS-1
AS151_R	CTCGCCATGGTTTGAGAATCAGCCATGCTT	to amplify 5'UTR of P*bclA* to create pAS-1
AS132_F	GCGCGAATTCCGATTGGCATTGATTTTCG	to amplify P*bclA*3 (172bp) of H111 to create pbdt-A3
AS133_R	GCGCCCATGGTTTGAGAATCAGCCATGCTT	to amplify P*bclA*3 (172bp) of H111 to create pbdt-A3
AS79_F	GCCCTACACAAATTGGGAGA	pSU11
AS80_R	GACAGTATCGGCCTCAGGAA	pSU11
AS145_F	GCATCATATGTTCTTCGATGAGCTTAACG	to amplify internal fragment of I35_4766 for insertion to pSC200
AS146_R	CTGCTCTAGAGCGATACTCGTCGATCTG	to amplify internal fragment of I35_4766 for insertion to pSC200
AS140_F	ATCACGGCAGAAAAGTCCAC	pBAD24
AS141_R	CTGGCAGTTCCCTACTCTCG	pBAD24
AS142_F	GCATGAATTCACTATGTTCTTCGATGAGCTTAAC	to amplify I35_4766 for insertion to pBAD24
AS139_R	CGATAAGCTTACGGACATGATGGAAAAAC	to amplify I35_4766 for insertion to pBAD24
ES44_F	AGCTGATCCGGTGGATGAC	to test the plasmid pGPI-SceI used for in-frame deletions
ES45_R	ACGGTTGTGGACAACAAGC	to test the plasmid pGPI-SceI used for in-frame deletions
AS106_F	ACTACGACTCGGTCGACACC	flanking region of I35_4766, to test the mutant
AS87_F	TCTAGAACCGATTTTCGGAGGCTATT	flanking region of I35_4766, to test the mutant
AS90_R	GGTACCTCATGTCCGAAAACGAATCGAC	flanking region of I35_4766, to test the mutant
M13_F	TGTAAAACGACGGCCAG	
M13_R	CAGGAAACAGCTATGACC	
pSC200_F	GTCATACTGGCCTCCTGATGTCGTC	to test P_{rha} constructs

AS91_F	CAGATGATCGACGTGCTGAC	insertion mutant I35_4766
AS92_R	GGTGCCGCTGATCGTATAG	insertion mutant I35_4766
AS93_F	AAGCTTCTCCTCGAGCTGGCTCTTC	insertion mutant I35_2688
AS94_R	GGATCCAGACGTACGATCTGCTGCTG	insertion mutant I35_2688
AS138_F	GACTGCTAGCCGCCGGATAACCATAATGT	to amplify I35_4766 for insertion to pBAD24

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Chapter 5

The DSF type quorum sensing signalling system RpfF/R regulates diverse phenotypes in the opportunistic pathogen *Cronobacter*

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5.1 Abstract

Several bacterial pathogens produce **d**iffusible **s**ignal **f**actor (DSF)-type quorum sensing (QS) signals to control biofilm formation and virulence. Previous work showed that in *Burkholderia cenocepacia* the $RpfF_{Bc}$/RpfR system is involved in sensing and responding to DSF signals and that this signal/sensor gene pair is highly conserved in several bacterial species including *Cronobacter* spp. Here we show that *C. turicensis* LMG 23827^{T} possesses a functional RpfF/R system that is involved in the regulation of various phenotypes, including colony morphology, biofilm formation and swarming motility. *In vivo* experiments using the zebrafish embryo model revealed a role of this regulatory system in virulence of this opportunistic pathogen. We provide evidence that the RpfF/R system modulates the intracellular c-di-GMP level of the organism, an effect that may underpin the alteration in phenotype and thus the regulated phenotypes may be a consequence thereof. This first report on an RpfF/R-type QS system of an organism outside the genus *Burkholderia* revealed that both the underlying molecular mechanisms as well as the regulated functions show a high degree of conservation.

5.2 Introduction

Members of the genus *Cronobacter* spp. are considered opportunistic pathogens associated with rare but severe neonatal systemic infections predominantly in pre-term and/or low birth weight infants and thus have attracted the attention of public health authorities and researchers in the past (Gurtler *et al.*, 2005; Bowen and Braden, 2006; Iversen *et al.*, 2008).

Epidemiological investigation of outbreaks of *Cronobacter* spp. infections in hospitals indicated powdered infant formula as a source of contamination, when these organisms were isolated from both reconstituted milk as well as from milk feeding equipment and utensils. The latter may be enhanced by the organism's ability to adhere and form biofilms on many surfaces, including silicone, latex, polycarbonate (used in the feeding bottle manufacture) and stainless steel (Iversen and Forsythe, 2003; Lehner *et al.*, 2005).

Bacterial processes involved in biofilm formation and virulence, are often controlled by quorum sensing (QS), a mechanism based on the production, release and detection of signalling molecules of low molar mass. Extracellular concentrations of signal molecules are sensed by the bacteria and, upon reaching a population density-dependent threshold, they are detected by the cells, which in turn induce target gene expression in a coordinated

fashion (Whitehead *et al.*, 2001; Waters and Bassler, 2005; Reading and Sperandio, 2006; Ryan and Dow, 2008).

To date many structurally unrelated signal molecules have been identified, including *N*-acyl-homoserine lactones (AHLs) in Gram-negative bacteria, oligopeptides in many Gram-positive bacteria and autoinducer-2 (AI-2), which is thought to serve as a signal for interspecies communication (Waters and Bassler, 2005; Reading and Sperandio, 2006; Ryan and Dow, 2008).

Another group of signal molecules are the *cis*-2-unsaturated fatty acids, often referred to as DSF (**d**iffusible **s**ignal **f**actor) family signals (Deng *et al.*, 2011). The first fatty acid signal, *cis*-11-methyl-2-dodecenoic acid, was identified in the culture supernatant of the phytopathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Barber *et al.*, 1997). Subsequently, fatty acid-based QS-systems were also identified and in members of the genera *Xylella* and *Stenotrophomonas* (Fouhy *et al.*, 2007; Ham, 2013) where they were shown to control the production of virulence factors (Barber *et al.*, 1997). More recent work showed that *Burkholderia cenocepacia* produces the signal molecule *cis*-2-dodecenoic acid, which was named BDSF (***B**urkholderia* **d**iffusible **s**ignal **f**actor) (Boon *et al.*, 2008). BDSF is synthesized by the enoyl-CoA hydratase $RpfF_{Bc}$ (Bi *et al.*, 2012) and is sensed by the receptor protein RpfR, which contains PAS-GGDEF-EAL domains (Deng *et al.*, 2012). Binding of BDSF to the PAS domain stimulates the c-di-GMP phosphodiesterase activity of RpfR, which in turn lowers the intracellular c-di-GMP level. This signal transduction relay is very different from the one originally described for *X. campestris*, in which the DSF receptor RpfC is a hybrid sensor kinase that phosphorylates its cognate response regulator RpfG. This regulator contains in addition to a REC domain a HD-GYP domain, which is responsible for the c-di-GMP phosphodiesterase activity of the protein (Ryan *et al.*, 2006).

Interestingly, homologs of $RpfF_{Bc}$ and RpfR are present not only in many *Burkholderia* species but also in strains belonging to the genera *Achromobacter*, *Yersinia*, *Serratia*, *Enterobacter* and *Cronobacter* (Deng *et al.*, 2012), suggesting that RpfF/R type signaling systems may by far more widespread than anticipated. In this study we analysed the RpfF/R system of the clinical strain *Cronobacter turicensis* LMG 23827^T, and show that it is involved in the regulation of biofilm formation, macrocolony morphology, proteolytic activity and virulence.

5.3 Results and Discussion

5.3.1 RpfF directs the synthesis of a DSF family signal molecule and negatively regulates intracellular c-di-GMP levels in *C. turicensis*

Previous work identified homologs of both RpfR and RpfF from *B. cenocepacia* in *C. turicensis* LMG 23827^{T} (Deng *et al.*, 2012). To investigate the role of this putative QS system in this organism we constructed defined mutants as well as genetically complemented derivatives thereof. We tested the strains for the production of DSF family signal molecules by the aid of the *Burkholderia*-based biosensor H111–rpfF$_{Bc}$ pAN-L15 both in cross-streaking and liquid culture experiments. Under the conditions tested the wild type strain did not induce the biosensor. However, the complemented *rpfF* mutant, in which the wild type allele is expressed from a plasmid, clearly induced the biosensor (Figure 5.1), suggesting that RpfF directs the biosynthesis of a *cis*-2 fatty acid signal molecule. We hypothesize that under standard laboratory conditions the amount of signal released by the wild type strain is below the detection limit of our bioassay but that the complemented strain, in which *rpfF* is expressed from a plasmid, produces sufficiently high amounts to induce the biosensor.

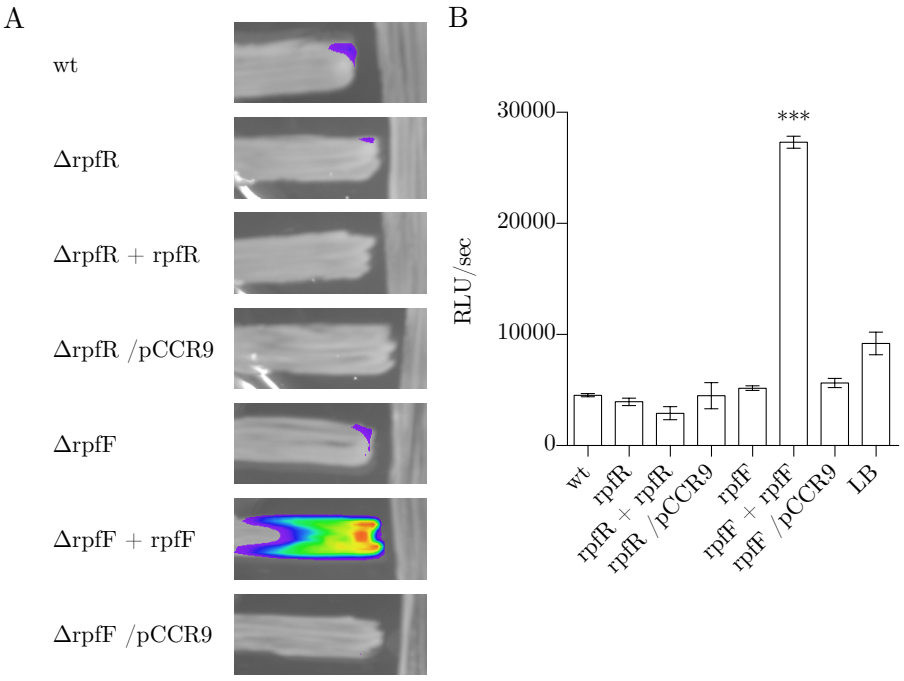

Figure 5.1 Overexpression of *rpfF* activates the biosensor *B. cenocepacia* H111-rpfF$_{Bc}$ / pANL15, which is capable of detecting various DSF family signals. **A**, The *C. turicensis* LMG 23827^{T} wild type (wt), the mutants (Δ*rpfF*, Δ*rpfR)*, the complemented mutants (Δ*rpfF* + *rpfF*, Δ*rpfR* + *rpfR*) and the mutants carrying the empty vector (Δ*rpfF* / pCCR9, Δ*rpfR* / pCCR9) (vertical) were tested in cross-streak experiments against the biosensor (horizontal). The biosensor was clearly induced by the complemented *rpfF* mutant (Δ*rpfF* + *rpfF)*. **B**, The strains were also tested for the production of DSF family molecules in liquid assays. As with the cross streaking, induction of the biosensor was only observed with the Δ*rpfF* + *rpfF* strain. Error bars indicate SEM, n=4; * P<0.05 (ANOVA, oneway).

RpfR family proteins contain a GGDEF as well as an EAL domain which are associated with the synthesis and degradation of c-di-GMP respectively (Römling *et al.*, 2005). The *C. turicensis* LMG 23827^{T} RpfR homolog CBA31265 exhibits an identical domain structure. In order to evaluate the role of RpfR in this strain the intracellular c-di-GMP levels were determined in the wild type, the *rpfR* and *rpfF* mutants and in the complemented strains Δ*rpfR* + *rpfR* and Δ*rpfF* + *rpfF*. The intracellular c-di-GMP level of the *rpfR* and *rpfF* mutants was found to be 3.9-fold or 3.4-fold increased relative to the wild type. Genetic complementation of the mutant reduced the c-di-GMP level to the level of the wild type. These results suggest that both RpfR and RpfF have a negative effect on the intracellular c-di-GMP level (Figure 5.2). This is in agreement with the finding that RpfR in *B. cenocepacia* exhibits a net phosphodiesterase activity (Deng *et al.*, 2012).

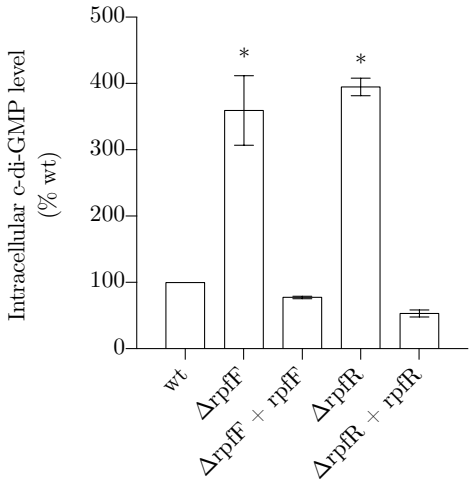

Figure 5.2 RpfR and RpfF affect the intracellular c-di-GMP level. The intracellular c-di-GMP level was significantly increased in the *rpfR* and *rpfF* mutants relative to the wild type. Detection was performed by by LC-MS/MS. Error bars indicate SEM, n=2; * P<0.05 (ANOVA, oneway).

5.3.2 RpfF/R plays a role in quorum sensing regulated phenotypes in *C. turicensis* LMG 23827^T

We next investigated whether the RpfF/R system is involved in the regulation of typically QS-associated phenotypes. In contrast to the wild type we observed a rough colony morphology of the *rpfR* and to a lesser degree with the *rpfF* mutant on Congo red agar plates (Figure 5.3). The strong pinkish colour of the *rpfR* mutant relative to the wild type may suggest an increased production of cellulose and/or curli (Römling *et al.*, 1998). In order to support this hypothesis, we performed expression studies targeting the gene coding for the catalytic subunit of the cellulose synthase *bscA* as well as the major curli subunit *csgA*. Expression of both genes was considerably increased in the mutants compared to the wild type. However, complementation only partially restored their expression (Figure 5.4).

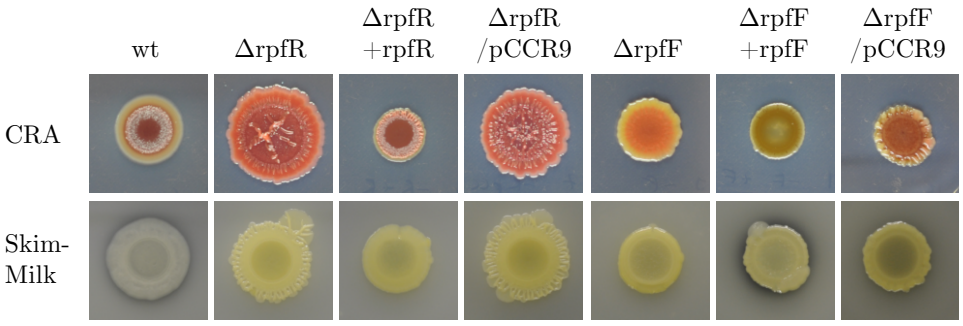

Figure 5.3 The RpfF/R QS-system controls colony morphology and protease production. Deletion of *rpfR* induced a rough, wrinkly colony morphology and increased EPS production on Congo red agar plates (CRA, upper panel). Both the Δ*rpfR* and the Δ*rpfF* mutant showed reduced protease production on skim-milk plates compared to the wild type (lower panel).

In addition, we observed that both mutants showed reduced proteolytic activity (Figure 5.3). Swarming motility on NYG + 0.4 % agar was not significantly affected by inactivation of the RpfF/R system. However, the complemented mutants exhibited increased swarming motility (Figure 5.5). These results are supported by the results of the RT qPCR experiments targeting the flagellar regulon-associated gene *flhE*, which was unaltered in the *rpfF/R* mutants but significantly higher in the complemented mutants (Figure 5.4). This finding may be explained by a dose effect due to the additional copies of this gene in the complemented mutants.

Both mutants formed significantly more biofilm under static conditions in microtiter plates (Figure 5.6A) than the parental strain. Complementation of the mutants partially restored the wild type phenotype. In the study by Hartmann *et al.* (2010) (Hartmann *et al.*, 2010) genes involved in biofilm formation in the closely related species *Cronobacter sakazakii* were identified using a transposon mutagenesis approach. *BscA* and *flhE* were – amongst others – two of the genes that were found to contribute to biofilm formation. Our expression analysis performed in this study suggests that *bcsA* but not *flhE* is regulated by the rpfF/R system (Figure 5.4).

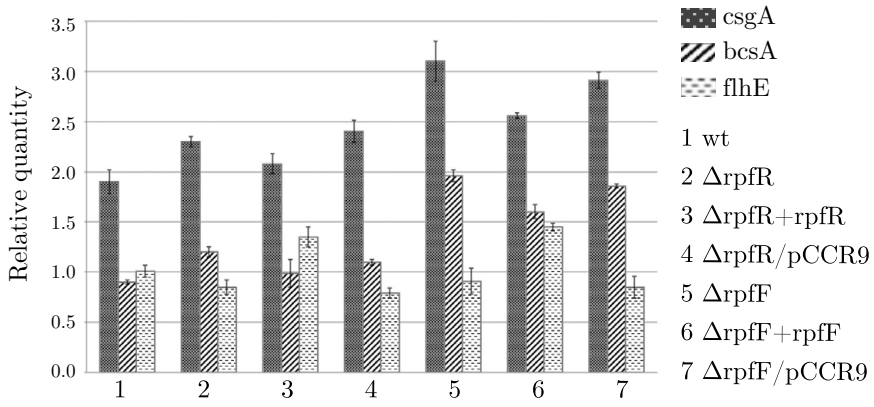

Figure 5.4 RT-qPCR analysis of *csgA*, *bcsA* and *flhE* gene expression in *C. turicensis* LMG 23827^{T} (wt), the mutants (Δ*rpfF*, Δ*rpfR*), the complemented mutants (Δ*rpfF* + *rpfF*, Δ*rpfR* + *rpfR*) and the mutants carrying the empty vector (Δ*rpfF* / pCCR9, Δ*rpfR* / pCCR9). The respective mRNA levels were normalized to the 16S rRNA reference gene. Error bars indicate SEM, n = 3.

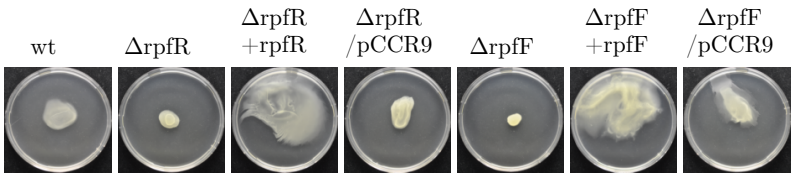

Figure 5.5 Overexpression of the RpfF/R QS-system increased swarming motility. Strains were spot inoculated on 0.4 % NYG agar and plates were photographed after 24h incubation.

Importantly, the strains Δ*rpfR* + *rpfR* and Δ*rpfF*/pCCR9 showed growth defects and did not reach the same OD as the other strains, which may explain the poor complementation of the Δ*rpfR* mutant. The growth curves of wild type strains, complemented mutants and mutants carrying the pCCR9 vector are depicted in Supplementary Figure S 5.1. Partial restoration was also observed when the *rpfF* mutant was supplemented with at least 1 µM BDSF or 20 µM DSF (Figure 5.6B). We also tested the various strains for pellicle formation, i.e. biofilm formation at the liquid-air interface. Both mutants showed increased pellicle formation and complementation restored the wild type behavior (Figure 5.6C). In a study by Lehner *et al.* (Lehner *et al.*, 2005) it has been reported that cellulose is one of the major components present in pellicles formed in *Cronobacter* spp. strains. The increased expression levels of *bcsA* in the mutants as observed in our study suggest a negative influence of the RpfF/R regulon in *C. turicensis* biofilm formation. This is in contrast to the homologous system of *B. cenocepacia* (Deng *et al.*, 2012) but similar to the genetically different DSF-dependent RpfCG systems of *Stenotrophomonas maltophilia* E77 or *X. campestris* pv. *campestris* (Crossman and Dow, 2004; Huedo *et al.*, 2014).

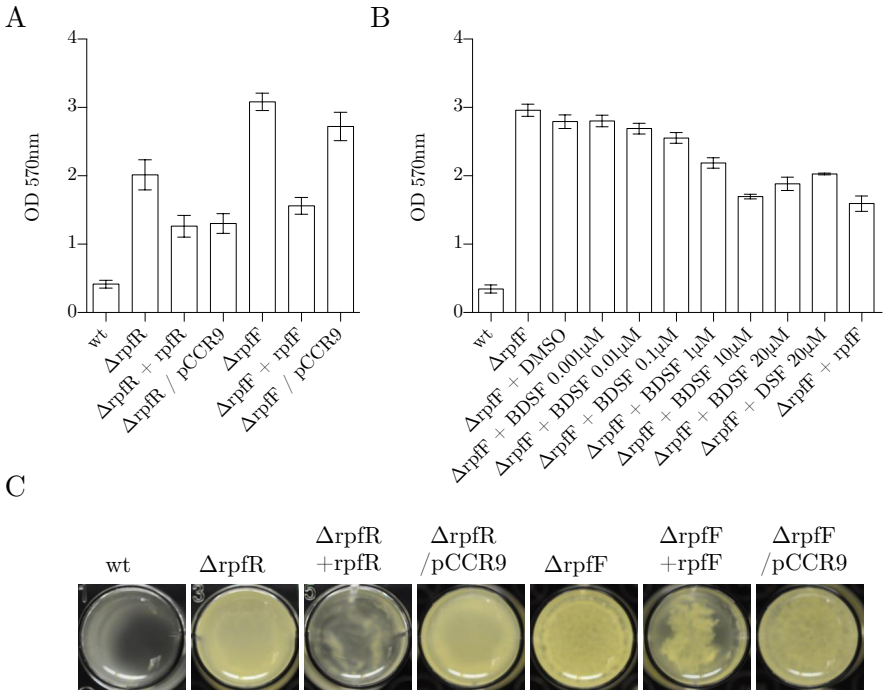

Figure 5.6 The RpfF/R system regulates biofilm formation under static conditions. A, Deletion of either *rpfR* or *rpfF* increased biofilm formation in microtiter plates. **B**, Partial restoration was obtained by genetic complementation or by supplementing the medium with BDSF (0.001 µM-20 µM) or DSF (20 µM). Error bars indicate SEM, n>2. (c) Both RpfR and RpfF are involved in pellicle formation tested in NYG broth at room temperature for 48h.

5.3.3 Zebrafish infection studies

We tested the Δ*rpfF* and the Δ*rpfR* mutants for pathogenicity in a zebrafish infection model. The dsRed-labeled wild type strain *C. turicensis* LMG23827^T (wt::dsRed) served as control. The mortality rate of the zebrafish larvae at 48 hpi increased to approximately 90 % for injection with wt::dsRed whereas the mortality rate decreased to 50 % when the larvae were infected with the mutant Δ*rpfF* (Figure 5.7A). Furthermore, the bacterial load was significantly lower with the *rpfF* mutant when compared with the wild type control (Figure 5.7B) indicating a role of the RpfF/R system in the expression of virulence factors required for pathogenicity in the zebrafish model. Injection experiments using the complemented mutant strain (Δ*rpfF* + *rpfF*) resulted in higher mortality rate and higher bacterial load, whereas control experiments using the mutant strain transformed with the vector alone (Δ*rpfF* / pCCR9) yielded mortality rates and bacterial loads comparable to the ones observed in the Δ*rpfF* mutant experiments (data not shown).

Intriguingly, the mortality rate of larvae injected with the Δ*rpfR* strain was virtually indistinguishable from the wild type.

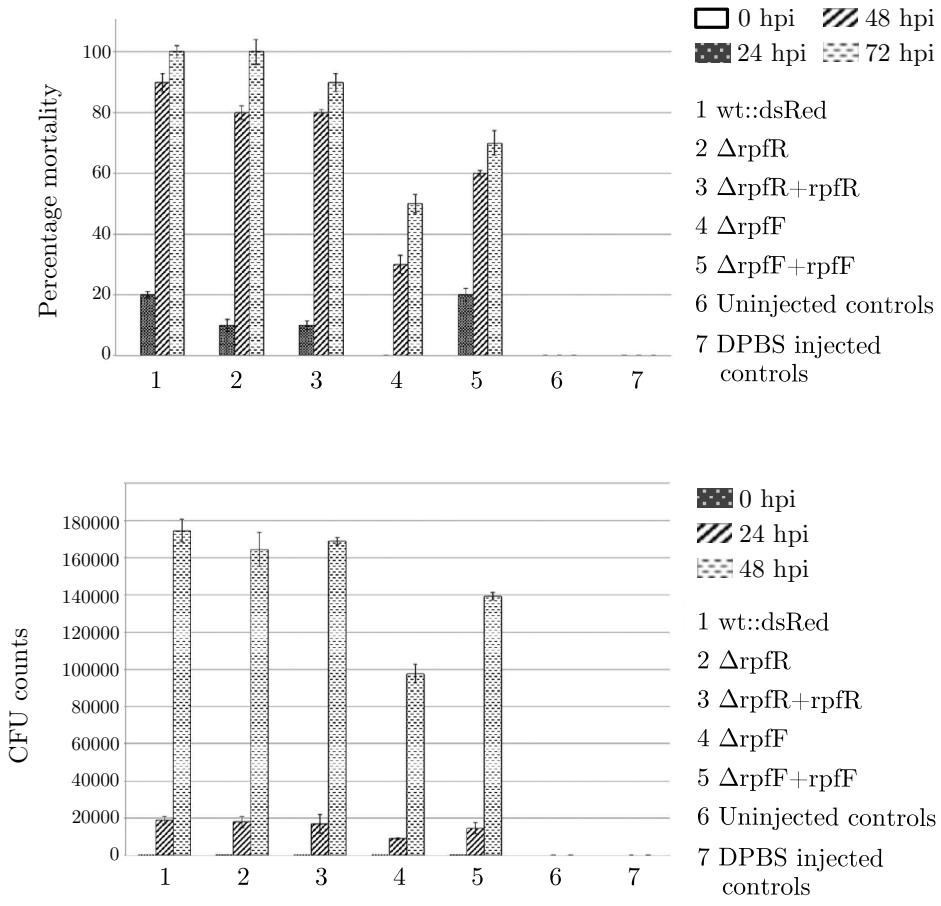

Figure 5.7 Survival rates of zebrafish larvae injected with 50 CFU *C. turicensis* LMG 23827^{T} / pRZT3::dsRed (wt::dsRed), the mutants Δ*rpfF* and Δ*rpfR* as well as the the complemented mutants Δ*rpfF* +*rpfF* and Δ*rpfR* +*rpfR*, **A**. A set of DPBS injected as well uninjected embryos served as controls. Error bars indicate SEM, n=3. **B**, Mean growth curve of *C. turicensis* LMG 23827^{T} / pRZT3::dsRED (wt::dsRed), the mutants Δ*rpfF* and Δ*rpfR* as well as the complemented mutants *(*Δ*rpfF* + *rpfF*, Δ*rpfR* + *rpfR)* inside infected zebrafish larvae with a starting inoculum of approx. 50 CFU. A set of DPBS injected as well uninjected embryos served as controls. Error bars indicate SEM, n = 3

Here we have shown that *C. turicensis* possesses a RpfF/R family QS system which relies on a *cis*-2-unsaturated fatty acid signal molecule. RpfF/R-type QS systems are particularly widespread among members of the genus *Burkholderia* (Deng *et al.*, 2011). We analysed for the first time a RpfF/R family QS system in a bacterium not belonging to the genus *Burkholderia* and demonstrated that despite the phylogenetic distance (β *versus* γ subdivision of Proteobacteria) both the molecular mechanism as well as the regulated phenotypes are very similar. Like in *B. cenocepacia*, the RpfF/R system was found to affect swarming motility, biofilm formation and virulence in *C. turicensis*. Furthermore, in both organisms the QS system modulates the intracellular secondary messenger c-di-GMP and this in turn appears to regulate the observed QS-dependent phenotypic traits. The finding that the *rpfF* but not the *rpfR* mutant reduced the virulence of *C. turicensis* suggests that an alternative signal receptor may be present in this strain. This is not unprecedented, as in *B. cenocepacia* an alternative BDSF receptor, BCAM0227, has been identified that is used by some strains as a parallel signaling system to control a subset of functions (McCarthy *et al.*, 2010). However, a bioinformatic analysis neither identified a homolog of BCAM0227 nor of *rpfC*, the DSF receptor of *Xcc* (He *et al.*, 2006).

In conclusion, our data provide evidence that RpfF/R-type QS systems are not restricted to *Burkholderia* sp. but may be widespread among Gram-negative bacteria, in which they influence surface colonization and virulence through modulation of the intracellular c-di-GMP levels. It will be of interest to investigate if homologous systems in other bacteria will control the same phenotypes.

5.4 Material and Methods

Bacterial strains and culture conditions

C. turicensis LMG 23827^{T} (Stephan *et al.*, 2011), a clinical isolate responsible for two fatal sepsis cases in neonates in Zurich in 2006 was used in the study. Strains *C. turicensis* LMG 23827^{T}_Nal^{R} as well as *C. turicensis* LMG 23827^{T}/pRZT3::dsRed were described previously (Eshwar *et al.*, 2015; Fehr *et al.*, 2015).

For selection purposes, during zebrafish embryo infection experiments, *C. turicensis* LMG 23827^{T}_Δ*rpfF*/pCCR9 as well as *C. turicensis* LMG 23827^{T}_Δ*rpfR*/pCCR9 were constructed by transformation of the strains with the vector using standard methods.

Strains were grown in Luria–Bertani (LB) broth over night at 37 ºC with gentle shaking. Where appropriate, culture medium or agar was supplemented with nalidixic acid at 256 mg L^{-1} (*C. turicensis* LMG 23827^T_Nal^R), chloramphenicol at 30 mg L^{-1} (strains harbouring pDS132) or both (transconjugant strains) or tetracyclin at 50 mg L^{-1} (strains harbouring pCCR9 or pRZT3::dsRed).

For microinjection experiments, the bacteria were harvested by centrifugation at 5000 x *g* for 10 min and washed once in 10 ml of Dubelcco's phosphate buffered saline (DPBS, Life Technologies, Switzerland.) After a second centrifugation step, the cells were resuspended in DPBS, and appropriate dilutions were prepared in DPBS.

DNA extraction and manipulations

Chromosomal DNA was isolated using the DNeasy Blood and Tissue kit, plasmids were extracted with the QIAprep Spin Miniprep or Plasmid Midi kits following the manufacturer's instructions. For purification purposes (PCR, restriction digest, agarose gel purification) the Qiagen MinElute PCR Cleanup kit or MinElute Gel Purification kit was employed. Enzymes and respective buffers were obtained from Roche Molecula Diagnostics (Rotkreuz, Switzerland) and used according to the manufacturer's instructions.

Construction of *C. turicensis* LMG 23827^T in-frame deletion mutants

Bacterial strains, plasmids and primers used for the construction of mutants are listed in Supplemental Table S 5.1. Deletion mutants of *C. turicensis* LMG 23827^T *rpfF* (CTU_23310) and *rpfR* (CTU_23300) genes were constructed following the protocol described by Philippe *et al*. (2004) (Philippe *et al.*, 2004). Details are provided in the supplementary material.

Phenotypic assays

Colony morphology: Overnight cultures grown in LB were adjusted to an OD_{600} = 1.0 in AB minimal medium (Clark and Maaloe, 1967). 5 µl of this cell suspension was spottet on CRA plates (2 g Casamino acids, 0.3 g yeast extract, 80 µl of 1 M $MgSO_4$, 4 g agar, dH_2O ad 200 ml, supplemented with 1.6 ml congo red (0.5 % in 50 % EtOH), 0.65 ml coomassie blue (0.3 % in 50 % EtOH). The plates were incubated at room temperature for six days before colonies were photographed.

Protease production: Cells of an overnight culture were resuspended in LB and 5 µl cell suspension was spottet on skim milk plates (1 % LB agar, 2 % w/v skim milk powder). Plates were incubated at 37 °C for two nights and then kept at room temperature.

Swarming motility: Analysis was performed as previously described by Deng *et al.* (2012) (Deng *et al.*, 2012), except motility was monitored on NYG plates containing 0.5 % peptone, 0.3 % yeast extract, 2 % glycerol and 0.4 % agar.

Biofilm formation: Overnight cultures were washed and diluted to an OD_{600} = 0.01 in AB minimal medium supplemented with 0.4 % glucose and 0.5 % casamino acids (Clark and Maaloe, 1967). 100 µl samples were added to 96 well plates incubated statically for 18 h at 30 °C. Growth was measured at 550 nM in a plate reader (Synergy HT; Bio-Tek, Germany). Surface attached cells were stained by the addition of 100 µl of 1 % crystal violet for 30 min at room temperature. The plate was washed thoroughly with tap water and air-dried. To solubilize the stain, 120 µl DMSO was added to each well, incubated for 20 min at room temperature and OD at 570 nm was measured. Data are based on at least 2 independent experiments with 7 technical replicates each.

Bioassays for the production of *cis*-2 fatty acids by using the biosensor *B. cenocepacia* H111-rpfF_{Bc}/pAN-L15. This sensor is sensitive to nM levels of synthetic BDSF and is suitable to detect a wide range of *cis*-2 fatty acid molecules (Suppiger *et al.*, submitted). In cross-streaking experiments both the test- and the sensor strain were streaked on LB agar plates close to each other to form a T. The plates were incubated overnight at 37 °C. Following the addition of 10 µl decanal to the lid of the plate the bioluminescence of the sensor strain was visualized using the NightOWL LB 983 (Berthold Technologies, Zug, Switzerland). In liquid bioassays the biosensor was grown in LB broth containing kanamycin 100 µg ml^{-1} to an OD_{600} of 2.0. Overnight cultures of the strains to be tested were centrifuged at 6000 rpm, 5 min and the supernatant (SN) was centrifuged again. 100 µl of this cell-free SN was mixed with 100 µl sensor and incubated for 20 h at 30 °C. Relative luminescence units (RLU) were obtained by adding 1-2 µl Decanal (Sigma Aldrich, Buchs, Switzerland) to each well and detection was performed using a plate reader (Synergy HT; Bio-Tek, Germany).

Intracellular cyclic-di-GMP level

Bacterial overnight cultures were subcultured in LB medium and 5 ml were harvested at an OD_{600} = 2.0 by centrifugation at 5000 rpm, 4 °C. Nucleotide extraction was performed as described by Spangler *et al.* (2010) (Spangler *et al.*, 2010) with slight modifications: cXMP

was omitted and the solvent was evaporated in Speedvac at 60 ºC. Quantification was performed by LC-MS/MS (Burhenne and Kaever, 2013).

Expression analysis of selected genes by RT-qPCR

The expression levels of the 16S rRNA, *csgA, bcsA,* and *flhE* genes in *Cronobacter turicensis* LMG23827^{T} wild type and its respective *rpfR* and *rpfF* mutants that were grown in AB medium supplemented with 0.4 % glucose and 0.5 % casamino acids at 30 ºC to early stationary phase were determined using reverse transcription quantitative-PCR (RT-qPCR). 1.5ml of the above bacterial suspension was re-suspended in 0.5 ml of the lysis buffer of the RNeasyPlus Mini Kit (Qiagen, Hilden, Germany). The samples were transferred on to the lysing bead matrix in MagNA lyser tubes and mechanically disrupted (1 min at 6500 rpm) using the MagNA Lyser Instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland). RNA was isolated from the bacterial lysates following the RNeasy^{Plus}Mini Kit protocol (Qiagen). Genomic DNA was removed by using a genomic DNA binding column and carrying out an on column DNAse I digestion. RNA was eluted in 50 µl of RNAse-free water, and subsequently quantified and quality controlled using the Nanodrop and BioAnalyzer instruments, respectively. 100 ng of RNA were reverse transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen). Residual DNA contamination was ruled out in each RNA sample by including a control in which the RT enzyme was omitted. Quantitative PCR was performed on 2.5 ng cDNA using the SYBR green I kit (Roche Molecular Diagnostics), and primers that are listed in Table S 5.2 in the LC480 (Roche Molecular Diagnostics) instrument. Following RT PCR conditions were applied for all four genes: 5 min 95 ºC followed by 40 cycles of 95 ºC for 10 seconds, 50 ºC for 20 seconds, 72 ºC for 20 seconds, 78 ºC for 1 second. Quantification was performed using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics). The *csgA, bcsA, flhE* mRNA levels were normalized using 16S rRNA as reference gene (Eshwar *et al.*, 2015).

Zebrafish infection studies

Zebrafish (*Danio rerio*) strains used in this study were albino lines. Husbandry, breeding and microinjection of approx. 50CFU of bacteria into the yolk sac of 2 dpf embryos was performed following the procedure described in the study by Fehr *et al.* (2015) (Fehr *et al.*, 2015).

A set of uninjected embryos, incubated in E3 maintenance medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) was included in order to determine the quality of the embryos; embryos injected with DPBS served as controls. Injected embryos were transferred into 24-well plates (1 embryo per well) in 1 ml E3 medium per well, incubated at 28 °C and observed for signs of disease and survival under a Leica M165 C stereomicroscope twice a day. In order to follow the course of infection embryos or larvae were collected at several time points, namely at 0, 24, 48 and 72 h post infection (hpi) and individually treated for bacterial enumeration.

Research was conducted with approval (NO 216/2012) from the Veterinary Office, Public Health Department, Canton of Zurich (Switzerland). The applied methods were carried out following the approved guidelines.

Bacterial enumeration by plate counting

The larvae were transferred to 1.5 ml centrifuge tubes and disintegrated by repeated pipetting and vortexing for 3 min in 1 mL of DPBS supplemented with 1 % Triton X-100 (Sigma-Aldrich, Buchs, Switzerland). Subsequently, serial dilutions of this mixture were plated onto LB plates supplemented with tetracycline 50 mg L^{-1} (strains harboring pCCR9 or pRZT3::dsRed). The plates were incubated up to 48 h at 37 °C.

Survival assay

Embryos were microinjected as mentioned above and maintained individually in 24-well plates in E3 medium at 28 °C. The number of dead larvae was determined at different time points visually based on the absence of a heartbeat.

Statistical analysis

Statistics and graphs were performed using GraphPad Prism 6 (GraphPad Software, San Diego, USA). Experiments were executed at least three times, unless stated otherwise. The CFU counts of individual larva at different time points and under different conditions were verified for significant variances by one-way ANOVA with Bonferroni's post-test.

Acknowledgments

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Author Contributions

A.S. and A.K.E. designed and conducted experiments along with data analyses and manuscript writing; V.K. performed cyclic-di-GMP measurements. L.E., A.L. and R.S. guided the work and evaluated manuscript and results; A.S. and A.K.E. equally contributed this work.

5.5 Supporting Information

Construction of *C. turicensis* LMG23827^T in-frame deletion mutants

Primers were designed based on the whole genome sequence of *Cronobacter turicensis* LMG 23827^T (RefSeq accession numbers NC_013282 to NC_013285, GenBank accession numbers FN543093 to FN543096). Briefly, two flanking fragments (upstream, downstream) of the *rpfF* and the *rpfR* genes were amplified by PCR using oligonucleotide primers rpfFmut1modf (containing a *XbaI* recognition site), rpfFmut2r (containing a *XhoI* restriction site), rpfFmut3f (containing a *XhoI* recognition site), rpfFmut5r (containing a *XbaI* recognition site), and rpfRmut1f (containing a *XbaI* recognition site), rpfRmut2r (containing a *XhoI* recognition site), rpfRmut3f (containing a *XhoI* recognition site), and rpfRmut5r (containing a *XbaI* recognition site), respectively. The amplification mixes contained 0.4 mM of primers, 1x AccuPrime (Invitrogen) buffer 2 (60 mM Tris-SO_4 (pH 8.9), 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 2 mM dGTP, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, thermostable AccuPrimeTM protein, 1 % glycerol), 4 % dimethylsulfoxid (DMSO), 2 U AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen) and 50 ng of template DNA. Following PCR conditions were used for the amplification: 95 °C for 120 s

followed by 34 cycles of 95 ºC for 30s, 68 ºC for 210 s and a final elongation step at 68 ºC for 300 s. The resulting fragments were digested with *XbaI* and *XhoI* and ligated into the suicide vector pDS132 digested with *XbaI*. The constructs pDS132::Δ*rpfF* and pDS132::Δ*rpfR* were transformed into *E. coli* SM10 λpir via electroporation. The resulting strains *E. coli* SM10 λpir/pDS132::Δ*rpfF* or *E. coli* SM10 λpir_pDS132::Δ*rpfR* served as donor strains for conjugative transfer of the plasmid into *C. turicensis* LMG 23827^{T}_Nal^{R}. Transconjugants were selected on LB agar plates supplemented with both nalidixic acid 256 mg L^{-1} and chloramphenicol 30 mg L^{-1}. The genetic structure of the mutants was confirmed by the presence of two amplification products - one representing the chromosomal wild type *rpfF* or *rpfR* allele and a second product representing the truncated (Δ*rpfF*, Δ*rpfR*) allele originating from the (integrated) pDS132::Δ*rpfF* or pDS132::Δ*rpfR* vector - after PCR using primer pair rpfFContf, rpfFConr, and rpfRConf, rpfRConr respectively employing the above mentioned AccuPrime amplification mixture (without DMSO) and following amplification conditions: 95 ºC for 120 s followed by 32 cycles of 95 ºC for 30 s, 54 ºC for 210 s and a final elongation step at 68 ºC for 300 s. The resulting amplification products were 825 bp (wt *rpfF* allele) and 110 bp (Δ*rpfF* allele) and 2185 bp (wt *rpfR* allele) and 217 bp (Δ*rpfF* allele) respectively.

Outcrossing was performed by plating serial dilutions of confirmed transconjugants onto LB agar plates supplemented with 5 % sucrose and no NaCl. Successful allelic exchange was verified in selected chloramphenicol sensitive and sucrose resistant strains by the presence of the mutant allele after PCR using the above mentioned procedure.

For constitutive expression (complementation) of *rpfF* and *rpfR* in the respective mutants, the genes were amplified with primers rpfFComplf, rpfFComplr or rpfRComplf, rpfRComplr using the above mentioned Accuprime mixture (without DMSO) and following conditions: 95 ºC for 120 s followed by 32 cycles of 95 ºC for 30 s, 58 ºC for 210 s and a final elongation step at 68 ºC for 300 s.

The amplicons were digested with *BamHI* and *HindIII* (for *rpfF* cloning) or *BamHI* and *XbaI* (for *rpfR* cloning) and ligated into low copy vector pCCR9 digested with the respective enzymes.

Supplemental Table S 5.1-S 5.2

Table S 5.1 Material used for mutant construction and complementation experiments.

Strains/plasmids/primers	Genotype/characteristic(s)/sequences	Source or reference
Mutant construction Strains		
C. turicensis LMG 23827^{T}_Nal^{R}	Acceptor for transconjugation, Nal^{R}	(Eshwar *et al.*, 2015)
E. coli SM10 λpir	Host for pDS132::Δ*rpfF*, pDS132::Δ*rpfR* construct generation; *thi, thr, leu, tonA lacY supE recA*::RP4-2-Tc::Mu, Km, λ*pir*	(Donnenberg and Kaper, 1991)
E. coli DH5α λpir / pDS132	Host for cloning vector pDS132; *sup* E44, Δ*lacU*169 (Φ80*lacZ*ΔM15), *recA1, endA1, hsdR17, thi-1, gyrA96, relA1,* λ*pir*, Cam^{R}	(Simon *et al.*, 1983)
E. coli SM10 λpir / pDS132::Δ*rpfF*	Donor for transconjugation, harbouring construct pDS132::Δ*rpfF*, Cam^{R}	This study
E. coli SM10 λpir / pDS132::Δ*rpfR*	Donor for transconjugation, harbouring construct pDS132::Δ*rpfR*, Cam^{R}	This study
Plasmids		
pDS132	Low copy cloning vector R6K *ori*, *mobRP4*, *cat*, *sacB*, CamR	(Philippe *et al.*, 2004)
pDS132::Δ*rpfF*	Δ*rpfF* cloned into pDS132, Cam^{R}	This study
pDS132::Δ*rpfR*	Δ*rpfR* cloned into pDS132, Cam^{R}	This study
Primers		
rpfFmut1modf	5'- ACC TCT AGA CAC GAC ACC ACT TCC GTG GCG - 3'	This study
rpfFmut2r	5'- TGA CTC GAG CGT AAT GAG CTG ATG GAA ATC ACC - 3'	This study
rpfFmut3f	5'- AGA CTC GAG AGT CTT TCC TCA TGT AAG TTA ACG - 3'	This study
rpfFmut5r	5' - TCG TCT AGA GGC GCT GCC GTC GGT CCA GGC - 3'	This study
rpfFConf	5' - CGC CGC GTC CAC CCA GTC - 3'	This study
rpfFConr	5' - ATT AGT CTG ATC CTG CGC - 3'	This study
rpfRmut1f	5' - AGT TCT AGA CGC CTG ACG CTG CTT ACG CAA ACC - 3'	This study
rpfRmut2r	5' - CCA CTC GAG GCG GCA GAC CCG GTG CGG TCA CGG - 3'	This study
rpfRmut3f	5' - GAC CTC GAG GTG GAG AGT GAA GAG GAA GAT GCG - 3'	This study
rpfRmut5r	5' - ACC TCT AGA GCC GGT TTC ATC AGC GGC GAA ATC - 3'	This study
rpfRConf	5' - GAG AAT TAA GCG AAA TGG - 3'	This study
rpfRConr	5' - GCG CTG CGT AAA GCA GGC - 3'	This study
Complementation		
C. turicensis LMG 23827^{T}	Template for amplification of *rpfF*, *rpfR* CDS	(Stephan *et al.*, 2011)
C. turicensis LMG 23827^{T}_ Δ*rpfF*	RpfF CDS mutant, cloning host for pCCR9, pCCR9::*rpfF*	This study
C. turicensis LMG 23827^{T}_Δ*rpfF* / pCCR9	Mutant transformant harbouring low copy cloning vector pCCR9, Tet^{R}	This study

C. turicensis LMG 23827^T_Δ*rpfF* / pCCR9::*rpfF*	Mutant transformant harbouring construct pCCR9::*rpfF*, Tet^R	This study
C. turicensis LMG 23827^T_ Δ*rpfR*	RpfR CDS mutant, cloning host for pCCR9, pCCR9::*rpfR*	This study
C. turicensis LMG 23827^T_Δ*rpfR* / pCCR9	Mutant transformant harbouring low copy cloning vector pCCR9, Tet^R	This study
C. turicensis LMG 23827^T_Δ*rpfR* / pCCR9::*rpfR*	Mutant transformant harbouring construct pCCR9::*rpfR*, Tet^R	This study
Plasmid		
pCCR9	Low copy cloning/expression vector, Tet^R	(Randegger *et al.*, 2000)
Primers		
rpfFComplf	5' - TTT GGA TCC GCA AAG GGA AAC AGA CGC - 3'	This study
rpfFComplr	5' - TTT AAG CTT GGT CAG CCA GCT GCT GCG - 3'	This study
rpfRComplf	5' - GTA GGA TCC AAA CCC GCA GCG TGA TCG - 3'	This study
rpfRComplr	5' - AAA TCT AGA CAG GCT AAC GGC CAT GAC - 3'	This study
pCCR9-F	5' - TTT GAC AGC TTA TCA TCG - 3'	(Schwizer *et al.*, 2013)
pCCR9-R	5' - CCT ATG GAA GTT GAT CAG - 3'	(Schwizer *et al.*, 2013)

Table S 5.2 Primers used for real time quantitative expression analysis.

Primers	Sequences	Source/Reference
Cturi_univ_16S_f	5`- GTG TTG TGA AAT GTT GGG T- 3`	(Eshwar *et al.*, 2015)
Cturi_univ_16S_r	5`- ACT AGC GAT TCC GAC TT- 3`	(Eshwar *et al.*, 2015)
csgA_f	5`- CGC AAT GGC ATT TCC T- 3`	This study
csgA_r	5`- GTT GTT GGC GAA ACC G-3`	This study
bcsA_f	5`- AAA GGG CTC AAG CTC G- 3`	This study
bcsA_r	5`- TTG GAG TTG GTC AGG C - 3	This study
flhE_f	5`- GCG CTA TGA ACT GGC A-3`	This study
flhE_r	5`- GCG GTA GTT GAC GAT G-3`	This study

Supplemental Figure S 5.1

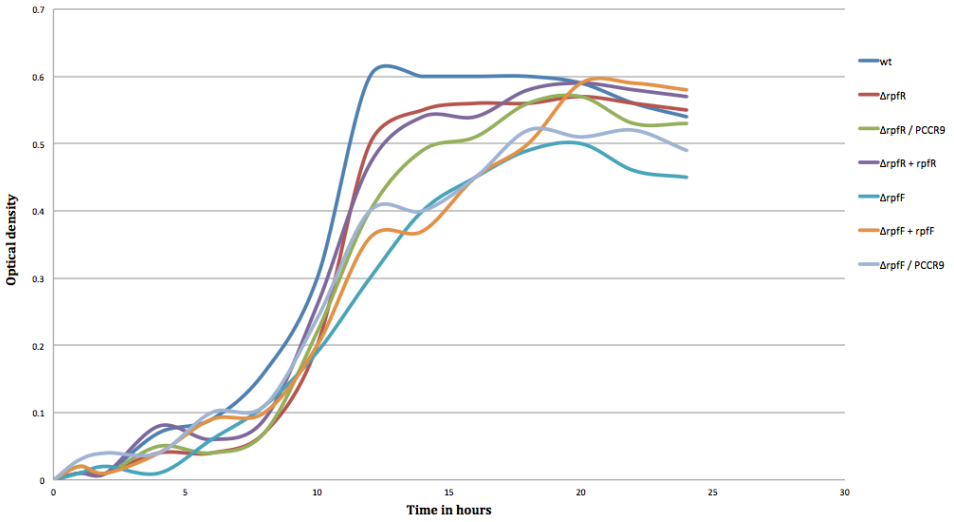

Figure S 5.1 Growth curves of *C. turicensis* wt, mutants and complemented mutants as well as mutants carrying the pCCR9 vector only in AB minimal medium supplemented with 0.4 % glucose and 0.5 % casamino acids. Bacterial growth was monitored over 24h at 30 °C and 600 nM in 200 µl volumes of medium supplemented in 96 well plates using the Bio-Tek microplate reader (Synergy HT; Bio-Tek, Germany).

Chapter 6

Conclusions and future perspectives

6.1 Fatty acid-based signalling is a widespread intra- and interspecies communication mechanism

6.1.1 Fatty acid-based signalling in *Burkholderia*

The novel *cis*-2 fatty acid biosensor *Burkholderia cenocepacia* H111-rpfF/pAN-L15 provides an easy tool for determining the ability of bacterial strains to synthesize DSF family signals. The biosensor was constructed on basis of the finding that the lectin operon *bclACB* is strictly BDSF- regulated (Schmid *et al.*, 2012). Expression of *bclACB* cannot be initiated by the AHL-based QS system alone, but is boosted when both the AHL- and BDSF-based systems are active (Schmid *et al.*, 2012). Thus, the lectin promoter provides a specific response to the BDSF-based signalling pathway. In contrast, the *bapA* promoter region was shown to be initiated by both BDSF- and AHL-based signalling pathways and was therefore not suitable for the construction of a BDSF specific biosensor (data not shown). The fusion of the *bclACB* promoter region to the reporter gene *luxAB* allowed detection of the signalling molecule BDSF in the nM range.

Detailed characterization of the biosensor revealed that expression of the reporter gene is not only inducible by BDSF, but also by DSF and *cis*-2 decenoic acid, suggesting specificity of the biosensor towards *cis*-2 fatty acids. It is noteworthy that *B. cenocepacia* was not found to produce DSF or *cis*-2 decenoic acid (however, some Bcc produce DSF) (Deng *et al.*, 2010). Whether the *B. cenocepacia* response to a variety of *cis*-2 fatty acids only plays a role under laboratory conditions, or if *B. cenocepacia* uses this as a means of interspecies communication in natural habitats requires further evaluation.

Due to its sensitivity and specificity for *cis*-2 fatty acids, the biosensor was used to identify bacterial strains that synthesize *cis*-2 fatty acids. We were able to show that 17 Bcc species synthesize *cis*-2 fatty acid molecules. Our experiments show that PBE *Burkholderia* species also synthesize this type of molecule, although in lower amounts compared to Bcc under the conditions tested. In general, fatty acid signalling molecules positively regulate virulence related factors (Ryan *et al.*, 2015). However, the plant pathogen *Xylella fastidiosa* has been shown to be more pathogenic upon interruption of its DSF-based signalling system (Newman *et al.*, 2004). Therefore, it would be particularly interesting to analyse the role of these signal molecules in non-pathogenic bacteria.

6.1.2 Fatty acid-based interspecies signalling

Burkholderia are mostly found in CF lungs in mixed populations with other bacterial strains (McKenney *et al.*, 1995). Furthermore, in the sputum of CF patients both DSF and BDSF signalling molecules were detected at physiologically relevant levels (Twomey *et al.*, 2012). Owing to these findings it has been suggested that interspecies signalling between *Pseudomonas* and *Burkholderia* might occur (McKenney *et al.*, 1995) (Riedel *et al.*, 2001). Indeed, *P. aeruginosa* responds to DSF by persistence, polymyxin tolerance and altered biofilm formation (Ryan *et al.*, 2008; Twomey *et al.*, 2012).

In *B. cenocepacia* DSF can substitute for BDSF (Deng *et al.*, 2012) and it has also been shown that BDSF is able to activate DSF-dependent responses in *X. campestris*, suggesting that cross-species signalling between these organisms is possible (Boon *et al.*, 2008). Overall, it would be exciting to extend these findings; to look for co-occurrence of bacterial species in different habitats, to characterize the physiological conditions and timing required for signal molecule production or response, the diffusion range as well as physiological concentrations of the signals.

6.1.3 Fatty acid-based signalling in *Cronobacter*

The RpfF/R-based QS system in *Cronobacter turicensis* was characterized in within this thesis. For the first time, we have demonstrated a regulatory role of the RpfF/R system in an γ-Proteobacterium. Both RpfF and RpfR were shown to play a role in biofilm formation, colony morphology, protease production and swarming motility in *C. turicensis*. Furthermore, under the conditions tested RpfR was found to exhibit a net phosphodiesterase activity, similar to the situation in *B. cenocepacia*. The results suggest that the RpfF/R QS system is a core system for fatty acid-based signalling perception. However, more work is required to fully characterize the RpfF/R QS system in *Cronobacter*. It is worth noting that exogenous addition of purified DSF or BDSF to an *rpfF* mutant did not fully complement all phenotypes to wild-type level (e.g. for biofilm formation). Furthermore, the *C. turicensis* wildtype was found to induce the *cis*-2 fatty acid sensitive biosensor only when the *rpfF* mutant was complemented with the wild-type allele *in trans*. Therefore, for future studies it will be of interest to identify the fatty acid signalling molecule from the wildtype strain and to evaluate the optimal culture conditions for its synthesis.

6.1.4 Factors that influence production of the fatty acid signal molecule

My investigations on *cis*-2 fatty acid production was performed using LB as the growth medium. It has been shown that the precise structure of the DSF-family signal synthesised depends on the culture medium composition (Deng *et al.*, 2010; He *et al.*, 2010; Zhou *et al.*, 2015). For example, *Xanthomonas campestris* pv. *campestris* (*Xcc*) mainly produces BDSF in the presence of carbohydrates, but DSF in the presence of leucine (Zhou *et al.*, 2015). Depending on the nutrient source, different types of acyl-ACPs are produced, which in turn are modified by the fatty acid synthesis elongation cycle and then serve as precursors for the DSF-family signal synthesis by RpfF. RpfF is thought to have non-specific thioesterase activity and to cleave a wide variety of acyl-ACPs in both *Burkholderia* and *Xcc* (Bi *et al.*, 2012; Zhou *et al.*, 2015). The presented data provide evidence that the medium composition influences the stoichiometric composition of the signal molecules. However, in *Burkholderia* it has been shown that the genetic background also influences the type of DSF signal molecule produced (Deng *et al.*, 2010).

6.2 Fatty acid-based signalling as a target for virulence control

To date, fatty acid-based intraspecies signalling has been identified in various bacteria, including *Burkholderia*, *Cronobacter*, *Xanthomonas*, *Xylella*, *Pseudomonas* and *Stenotrophomonas* (Ryan *et al.*, 2015; Suppiger *et al.*, 2016b). The widespread occurrence of DSF-based signalling in intraspecies-, and interspecies communication makes this QS system a potential target for pathogenicity control.

The study of QS systems began in the late 1960s (Kempner and Hanson, 1968; Nealson *et al.*, 1970), and the first autoinducer determined was the AHL molecule 3-oxo-C6-HSL (Eberhard *et al.*, 1981). Thus, the AHL-based QS system has been characterized for many years, its conservation among numerous Gram-negative bacteria has been shown and several methods to interfere with this communication mechanism have been described. For example, AHL signalling molecules can be degraded by AHL-acylases or AHL-lactonases, which are produced by several bacterial species; an approach designated 'quorum quenching' (Gonzalez and Keshavan, 2006; Dong *et al.*, 2007). Recently, a range of small-molecule non-native agonists and antagonists of the QS receptor RhlR in *P. aeruginosa* were investigated for their effect on virulence (Eibergen *et al.*, 2015; Xu *et al.*, 2016).

The DSF-family signalling systems have also been targeted to interfere with the pathogenic potential of the producing strains, in a manner analogous to quorum quenching. A variety of DSF degrading bacteria have been isolated from various plant-associated bacteria (Newman *et al.*, 2008). It has been found that DSF degradation is a common bacterial trait, but few strains are capable of very efficient DSF degradation. For example, *Bacillus*, *Staphylococcus* and *Pseudomonas* spp. have been shown to exhibit particularly fast DSF degradation rates (Newman *et al.*, 2008). The molecular mechanism is thought to be based on structural DSF modifications, which depend on an enzymatically highly active carbamoylphosphate synthetase, encoded by *carAB*.

Another approach to interrupt fatty acid-based bacterial communication is to interfere with the signalling system using structural DSF-analogues. For example, BDSF was shown to inhibit *Candida albicans* adherence to catheters (Tian *et al.*, 2013), DSF was shown to enhance the antimicrobial efficacy of antibiotics against *Bacillus* and other bacterial pathogens (Deng *et al.*, 2014) and *cis*-2 decenoic acid reduced biofilm formation in several bacterial species (Sepehr *et al.*, 2014) (Jennings *et al.*, 2012) or revert persister cells to a susceptible state (Marques *et al.*, 2014).

QS is a tightly controlled mechanism, therefore the consequences of over-production of signalling molecules on virulence were studied (Caserta *et al.*, 2014). Plants were engineered to express *rpfF* thereby increasing DSF-levels. This in turn reduced virulence of the plant pathogen *Xanthomonas* to the transgenic plant host (Caserta *et al.*, 2014).

6.3 Mechanism of fatty acid-based signalling systems

DSF signal perception and transduction in *Xcc* was shown to be conferred by the RpfC/RpfG two-component regulatory system, as described in Section 1.3.2 (Slater *et al.*, 2000). In both *B. cenocepacia* and *Xcc*, DSF-family based signalling is involved in c-di-GMP turnover (Ryan *et al.*, 2006; Ryan *et al.*, 2015). In *Xcc* a second DSF-sensor is known; RpfS is a histidine sensor kinase with a DSF-binding PAS domain, that controls a subset of the DSF-regulated genes (An *et al.*, 2013). On the other side, in *B. cenocepacia* a second sensor has been described; the sensor kinase BCAM0227 (McCarthy *et al.*, 2010). BCAM0227 is not a homolog of RpfC, and is involved in the regulation of only a subset of the BDSF-regulated genes. However, the widely conserved RpfR-RpfF and RpfF-RpfC-RpfG systems, are thought to constitute the core systems for DSF-family signalling and can occur together with other accessory sensor systems (Ryan *et al.*, 2015).

In my thesis I investigated the BDSF-regulated promoter region of *bclACB* in detail. Different promoter fragments were analysed for its activity in different mutant backgrounds. My analysis revealed that the BDSF-dependent signalling pathway includes the BDSF receptor RpfR, but not BCAM0227 and that the regulation mechanism is mediated via the 5' untranslated leader region of the target gene *bclA*. In addition, results suggest that *bclACB* expression is repressed in the absence of BDSF and thus an unknown BDSF effector may be required for de-repression. Further studies are required to analyse if this regulatory mechanism is specific for i) *bclACB* regulation, ii) a subset of BDSF-regulated genes or iii) all BDSF-regulated genes. A comparison of various BDSF-regulated promoters did not reveal a consensus sequence.

The lectin operon *bclACB* was shown to be primarily regulated by the BDSF-based system, but its expression can be boosted in the presence of AHLs. We propose that both systems control gene expression via distinct sequences upstream of the translational start site. The presence of two QS systems allows a fine tuned regulation of gene expression. Specific environmental conditions and stoichiometric balance of cellular components might influence the response to both BDSF and AHLs. The underlying reasons and mechanism require further evaluation.

6.4 Role of LepR as a novel regulator in *B. cenocepacia*

In this study, LepR (I35_4766) was found to be involved in the regulation of the lectin operon *bclACB*, the production of the EPS cepacian and other virulence-related phenotypes. We propose that LepR is a global transcriptional regulator. For example, several genes involved in amino acid- and carbohydrate transport and metabolism were found to be regulated by LepR. Interestingly, H111-*lepR* was found to be more persistent to treatment with ciprofloxacin compared to the wildtype. These findings raise several new questions, that will be interesting to address in the future: Why does the same mutant show such diverse phenotypes ranging from abolished cepacian production and reduced lectin production to increased levels of carbohydrate transporters and the occurrence of more persister cells? LepR is an uncharacterized protein with no obvious domains that would provide hints on the underlying mechanism of this regulator.

As shown previously, *bclACB* expression was strongly reduced in an *rpfR* or a *lepR* mutant background. However, in both mutants weak *bclACB* expression was observed. Visual inspection revealed that this 'background' expression was differentially localized within

H111-rpfR and H111-lepR macrocolonies, suggesting that the RpfR mediated signalling pathway is active under different conditions than the LepR mediated induction.

Both the LepR- and the BDSF-based regulatory systems might be linked, as a *lepR* mutant expresses an increased level of carbohydrate transporters and BDSF production depends on carbohydrates (Deng *et al.*, 2010; Zhou *et al.*, 2015).

6.5 Regulation of the lectins BclACB in *B. cenocepacia*

In this thesis the expression of the lectin operon *bclACB* was shown to be regulated by several factors, including both BDSF- and AHL-based QS systems, but also environmental changes such as increased temperature, nitrogen- or oxygen-limitation (Inhülsen *et al.*, 2012; Schmid *et al.*, 2012; Pessi *et al.*, 2013; Lardi *et al.*, 2015). The lectins *bclACB* are transcribed as an operon (Inhülsen *et al.*, 2012). However, several transcriptomics studies have described only one or two of the lectins BclACB to be differentially expressed or the expression level between the individual lectins differed remarkably (O'Grady *et al.*, 2009; Schmid *et al.*, 2012; Pessi *et al.*, 2013). For example, only *bclA* and *bclB*, but not *bclC* were found to be regulated by CepR2 (O'Grady *et al.*, 2009). Therefore, regulation of *bclACB* expression requires further investigations. One interesting avenue of study might be the analysis of intercistronic regions or potential mRNA stabilisation mechanisms, that could be controlled by the BDSF-dependent signalling pathway. In this study, results have shown that BDSF-based regulation of *bclACB* is mediated by its 5' untranslated leader region. The 5' untranslated leader region can serve as a potential attack point for degradative nucleases, for ribosomes or it can be targeted by small non-coding RNAs (Rauhut and Klug, 1999; Ramos *et al.*, 2014). However, additional work will be required to test these possibilities.

6.6 Role of the lectins BclACB in *B. cenocepacia*

The lectin BclB has been found to play a role in virulence. The analysis of the purified proteins in virulence assays, have shown that pathogenicity towards the nematode *C. elegans* is mediated by the N-terminal domain of BclB, rather than by its mannose-binding C-terminal domain. In contrast, the mannose-binding domain of BclA (which is highly similar to the BclB C-terminal) was shown to bind bacterial surfaces, and was suggested to bind the LPS of bacteria (Lameignere *et al.*, 2008; Marchetti *et al.*, 2012). However, in *B. cenocepacia*

H111 no binding of BclA was observed to bacterial surfaces, but rather to fibre-like structures within bacterial clusters (Suppiger, 2012). These structures have not yet been identified, but due to their location, it is possible that they are EPS molecules. The EPS cepacian is built of several sugar moieties, including D-mannose. These results, as well as the finding of co-expression of cepacian with the lectin operon *bclACB*, suggest that the EPS cepacian, or the EPS molecule Bmp (a thus far uncharacterized EPS molecule (Fazli *et al.*, 2013)), are potential ligands of the lectins. This lectin-EPS complex in turn may be required for structural maintenance of biofilms. Both the lectins and Bmp have been shown to be required for biofilm formation in *B. cenocepacia* H111 (Inhülsen *et al.*, 2012; Fazli *et al.*, 2013).

6.7 Concluding Remarks

The aim of this PhD project was the further characterization of the BDSF-based QS system; to unravel its regulatory mechanism in *B. cenocepacia* H111 and to analyse the occurrence of DSF-family based signalling molecules in other bacterial species. By application of diverse molecular methods and phenotypic analyses, our understanding of the BDSF-based QS mechanism was deepened, and the wide conservation of *cis*-2 fatty acid-based signalling in *Burkholderia* species and in *Cronobacter turicensis* was demonstrated. This study identified a novel regulator (LepR) that links the production of the BclACB lectins with the biosynthesis of the EPS cepacian. With this thesis, two novel components were added to the the BDSF-based QS-signalling pathway in *B. cenocepacia*. However, it has also become apparent that the QS system is highly complexand further analysis is required to understand how the different components interact and how they influence pathogenicity of *B. cenocepacia*.

Appendix

A Virulence determinants of the Bcc species

Siderophores

Iron is an important trace element, required for bacterial survival and growth. Iron is one of the most abundant elements on earth. However, in the presence of oxygen and at neutral pH iron rapidly oxidises, from the ferrous redox state (Fe^{2+}) to the insoluble ferric form (Fe^{3+}). Therefore, in various ecological niches iron is often not readily available to bacteria (Guerinot and Yi, 1994; Andrews *et al.*, 2003). Bacteria employ different mechanisms to overcome iron limitation in the environment (Guerinot, 1994). One strategy to solubilize and to transport iron is the production and secretion of siderophores, low molecular weight molecules which chelate iron outside the cell and are subsequently taken up via specific transporters. Members of the Bcc are known to produce four different siderophores from the hydroxamate, catecholate and phenolate molecule classes; ornibactin, pyochelin, cepabactin and cepaciachelin (Thomas, 2007). Ornibactin, similar to pyoverdin of *P. aeruginosa*, is the predominant siderophore produced by Bcc species and has been shown to be a critical virulence determinant of *B. cenocepacia* (Darling *et al.*, 1998; Sokol *et al.*, 1999; Visser *et al.*, 2004; Uehlinger *et al.*, 2009). Pyochelin is produced in only about 50 % of clinical Bcc isolates, often strain specifically. For example, pyochelin is produced by *B. cenocepacia* H111, but not by *B. cenocepacia* J2315 or *B. cenocepacia* K56-2 (Darling *et al.*, 1998). Cepabactin was shown to be produced under iron-limited conditions by *B. cepacia* and the catecholate siderophore cepaciachelin is produced by the rhizosphere isolate *Burkholderia ambifaria* (Meyer *et al.*, 1989; Barelmann *et al.*, 1996). In the human host, *Burkholderia* species can use both heme and ferritin as iron sources (Whitby *et al.*, 2006; Thomas, 2007).

Protein secretion systems

Prokaryotes have evolved various ways to transport proteins, small molecules and DNA from the cytoplasm into other cell-compartments, the environment, other bacteria and eukaryotic cells. A number of Gram-negative bacteria rely on dedicated secretion systems to transport virulence proteins across bacterial membranes and sometimes directly into the cytoplasm of a target cell, where they influence host cell responses (Costa *et al.*, 2015; Green and Mecsas, 2016). Members of the Bcc employ different types of secretion systems dependent on the strain (Angus *et al.*, 2014). The following are the secretion systems of *B. cenocepacia*. *B. cenocepacia* has been found to have type I and type II secretion systems,

which secrete for example proteins with haemolytic and phospholipase activity, and the large surface protein BapA (Fehlner-Gardiner *et al.*, 2002; Latasa *et al.*, 2005). Furthermore, *B. cenocepacia* employs a type III secretion system, which is required for survival the murine agar bead infection model (Tomich *et al.*, 2003). In *B. cenocepacia* K56-2, two type IV secretion systems (T4SSs) have been identified. One of the systems transports plant cytotoxic proteins, causing disease in onions (Engledow *et al.*, 2004) and required for survival and replication in epithelial cells and macrophages (Sajjan *et al.*, 2008). The second T4SS is involved in plasmid mobilisation (Zhang *et al.*, 2009). A type VI secretion system (T6SS) has also been identified in *B. cenocepacia* and was shown be negatively regulated by the sensor kinase-response regulator AtsR (Aubert *et al.*, 2008). The T6SS has been shown to play a role in infection in the murine agar bead model (Hunt *et al.*, 2004), to protect from predation by the amoeba *Dictyostelium discoideum* and to be involved in actin rearrangements in macrophages (Aubert *et al.*, 2008).

Extracellular proteins (proteases, lipases and haemolysin)

Many Bcc species produce metallo- and serine proteases. Most Bcc members produce two zinc metalloproteases, ZmpA and ZmpB, that are secreted and have been associated with virulence (Corbett *et al.*, 2003; Gingues *et al.*, 2005). ZmpA and ZmpB are broad-spectrum proteases, that cleave for example type IV collagen, fibronectin and the α-1 protease inhibitor (Kooi *et al.*, 2005; Kooi *et al.*, 2006). Thereby, both proteases have the potential to cause tissue damage in the CF lung and to modulate the host immune system. ZmpA, but not ZmpB was found to be important for persistent respiratory infection in *B. cenocepacia* K56-2, however, expression and activity of ZmpA is strain specific (Corbett *et al.*, 2003).

Most Bcc strains produce lipases, that are required to provide free fatty acids to be used as an energy source (Lonon *et al.*, 1988; Carvalho *et al.*, 2007). Lipases have been shown to play a role in the invasion of epithelial cells (Mullen *et al.*, 2007) and persistence (Straus *et al.*, 1992).

Some Bcc isolates possess haemolytic activity (Carvalho *et al.*, 2007). For example, in *B. cenocepacia* the lipopeptide toxin haemolysin was identified, that is responsible for haemolysis of erythrocytes, induction of neutrophil apoptosis and degranulation of neutrophils (Hutchison *et al.*, 1998).

Surface structures (Pili, flagella)

Members of the Bcc express several surface structures including pili, flagella and adhesin that play important roles in motility and adherence to host cells (Urban *et al.*, 2005). Pili or fimbriae are proteinaceous surface appendages, that are responsible for attachment to a variety of surfaces such as epithelial cells and mucin. The Bcc species produce five morphologically distinct types of pili; the cable (Cbl) pilus, the filamentous (Fil) pilus, the spine (Spn) pilus, the spike (Spk) pilus and the mesh (Msh) pilus (Goldstein *et al.*, 1995). Attachment of Bcc to epithelial cells is mainly mediated by the cable pilus, which also contributes to persistence (Goldberg *et al.*, 2011). Therefore, cable pili play a role in virulence of *Burkholderia* (Sajjan *et al.*, 2002). However, not all *Burkholderia* species express cable pili. The expression of each pilus type has been linked to the sources from which the respective strains were isolated (CF associated vs. clinical but non-CF associated vs. environmentally associated isolates) (Goldstein *et al.*, 1995).

The flagellum is required for motility. *Burkholderia* with disrupted flagellum-specifying genes (*fliCII*) are attenuated for virulence in the mouse agar bead model of infection (Urban *et al.*, 2004).

Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is a glycolipid that consists of lipid A, a core oligosaccharide and a distal polysaccharide (O-antigen). Lipid A anchors the LPS to the outer membrane of Gram-negative bacteria, and confers general endotoxic activity (Raetz and Whitfield, 2002). The production of LPS by Bcc species has been shown to stimulate pro-inflammatory cytokines, resulting in the production of reactive oxygen species and thus causing tissue damage (Hutchison *et al.*, 2000; Bamford *et al.*, 2007). The lipid A of *B. cenocepacia* is highly acylated; a pattern that may contribute to the strong pro-inflammatory response (Silipo *et al.*, 2007).

LPS provides the bacteria an increased resistance to cationic peptides. For example, it has been shown that core oligosaccharide biosynthesis mutants of *B. cenocepacia* K56-2 are sensitive to polymyxin B (Ortega *et al.*, 2009). Furthermore, LPS mutants of Bcc species were attenuated for virulence in infection models such as rat agar bead, *Caenorhabditis elegans* and *Galleria mellonella* (Loutet *et al.*, 2006; Ortega *et al.*, 2009; Uehlinger *et al.*, 2009).

Exopolysaccharide (EPS)

Exopolysaccharides (EPS) are branched, repeating sugar-based subunits that are secreted by bacteria into the extracellular environment (Nwodo *et al.*, 2012). Members of the Bcc produce different EPS types, with some strains producing only a single type and others producing mixtures (Hallack *et al.*, 2010; Cuzzi *et al.*, 2014). In *Burkholderia*, the most commonly produced EPS is cepacian, a heptasaccharide repeating unit composed of 5 sugars; glucose, mannose, galactose, rhamnose and glucuronic acid (Ferreira *et al.*, 2010). Additional details of cepacian production in *Burkholderia* species are given in Chapter 4.

Production of EPS is necessary for the formation of mucoid colonies (Zlosnik *et al.*, 2008). Mucoid strains of *P. aeruginosa* form more biofilm, persist longer in the CF lung than non-mucoid strains and are linked to increased morbidity and mortality (Govan and Deretic, 1996; Jackson *et al.*, 2004). However, in *Burkholderia* the contribution of EPS to biofilm formation, virulence and persistence is highly strain and EPS type dependent (Huber *et al.*, 2002; Chung *et al.*, 2003; Cunha *et al.*, 2004; Zlosnik *et al.*, 2011).

Biofilm formation

In nature, most bacteria live as polymicrobial aggregates, for example in biofilms. Biofilms are surface-associated multicellular bacterial communities, in where the bacterial cells are embedded in self-produced extracellular polymeric substances. This matrix consists mainly of polysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender, 2010). Compared to planktonic cells, biofilm formation provides bacteria increased antibiotic resistance and a mechanism to evade the host immune system (Hall-Stoodley *et al.*, 2004). Members of the Bcc have been shown to form biofilms in *in vitro* experiments and to produce mixed biofilms in cultures with *P. aeruginosa*, another prevalent CF pathogen (Riedel *et al.*, 2001; Tomlin *et al.*, 2001; Conway and Greenberg, 2002; Bragonzi *et al.*, 2012). Growth of Bcc species in biofilms supports their persistence in CF lungs (Caraher *et al.*, 2007).

The formation of biofilms is a complex phenomenon, where bacteria adapt from free-living planktonic cells to a sessile multicellular lifestyle (Hall-Stoodley *et al.*, 2004; Monds and O'Toole, 2009). In *Burkholderia* many molecular mechanisms and factors that play a role in biofilm formation have been described (Fazli *et al.*, 2014).

B Abbreviations

°C	degree Celsius
ΔG	Gibbs free energy
ABC	AB-Citrate
ABG	AB-Glucose
ABM-ol	AB-Mannitol
ABM	AB-Mannose
ACP	acyl carrier protein
AHL	*N*-acyl-homoserine lactone
Amp	ampicillin
BDSF	*Burkholderia* diffusible signal factor, *cis*-2-dodecenoic acid
Bcc	*Burkholderia cepacia* complex
bp	base pair
C6-HSL	*N*-hexanoylhomoserine lactone
C8-HSL	*N*-octanoylhomoserine lactone
CAS	chrome azurol S
c-di-GMP	bis(3',5')-cyclic diguanylic acid
cDNA	complementary DNA
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CGD	chronic granulomatous disease
Cm	chloramphenicol
CRD	carbohydrate recognition domain
ddH_2O	double distilled water (Milli-Q water)
DGC	diguanylate cyclase
DPBS	Dubelcco\'s phosphate buffered saline
dH_2O	deionised water
DMF	dimethyl fumarate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxinucleotide triphosphate
DSF	diffusible signal factor
EDTA	ethylendiamintetraacetate
e.g.	exempli gratia, for example
EPS	exopolysaccharide
EtOH	ethanol

FL	follicular lymphoma
g	gram
GFP	green fluorescent protein
Gm	gentamicin
GMP	guanosine triphosphate
GNP	glycol-nanoparticle
h	hour
HHQ	2-heptyl-4-quinolone
hpi	hours post infection
i.e.	id est, that is
IPTG	isopropyl β -D-1-thiogalactopyranoside
kb	kilobases
kDa	kilodalton
Km	kanamycin
l	litre
LB	lysogeny broth (lennox), Luria-Bertani
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LPS	lipopolysaccharide
M	molar
MeOH	methanol
mg	milligram
min	minute
ml	millilitre
mM	millimolar
NB	nutrient broth
ng	nanogramm
NGM	nematode growth medium
nm	nanometer
nM	nanomolar
OD	opitical density
PBE	plant-beneficial-environmental
pC3	the former chromosome 3 of Bcc species
PCR	polymerase chain reaction
PDE	phosphodiesterase
pGpG	5'-phosphoguanylyl-(3'-5')-guanosine
PI	*Pseudomonas* isolation agar
pmol	picomole
PQS	*Pseudomonas* quinolone signal
QS	quorum sensing
RFU	relative fluorescence unit

RNA	ribonucleic acid
RNA-Seq	RNA sequencing
RPKM	reads per kilobase of transcript per million mapped reads
rpm	revolutions per minute
RT	room temperature
s or sec	seconds
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
spp.	species pluralis, multiple species
T4SS	type IV secretion system
T6SS	type VI secretion system
TCA	tricarboxylic acid
TLC	thin layer chromatography
Tp	trimethoprim
Tris	tris(hydroxymethyl)-aminomethan
R	resistance
UDP	uridine diphosphate
w/v	weight per volume
Xcc	*Xanthomonas campestris* pathovar *campestris*
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	microgram
µl	microliter
µM	mircomolar
µm	micrometer

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Curriculum Vitae

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Publications

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[1] equally contributed

Conference proceedings

Angela Suppiger, Claudio Aguilar, Leo Eberl. Development of a biosensor for investigating the BDSF quorum sensing system (Oral presentation) International *Burkholderia cepacia* working group (IBCWG), 18th annual meeting, Nimes, France, 2014

Angela Suppiger, Nadine Schmid, Elisabeth Steiner, Leo Eberl. The regulons of c-di-GMP and BDSF overlap in *B. cenocepacia* H111 (Poster) 1st International symposium on c-di-GMP signalling, Berlin, Germany, 2015

Angela Suppiger, Claudio Aguilar, Leo Eberl. Development of a biosensor for the detection of cell-to-cell communication molecules (Poster) 71th Annual assembly of the Swiss Society for Microbiology (SSM), Interlaken, Switzerland, 2013